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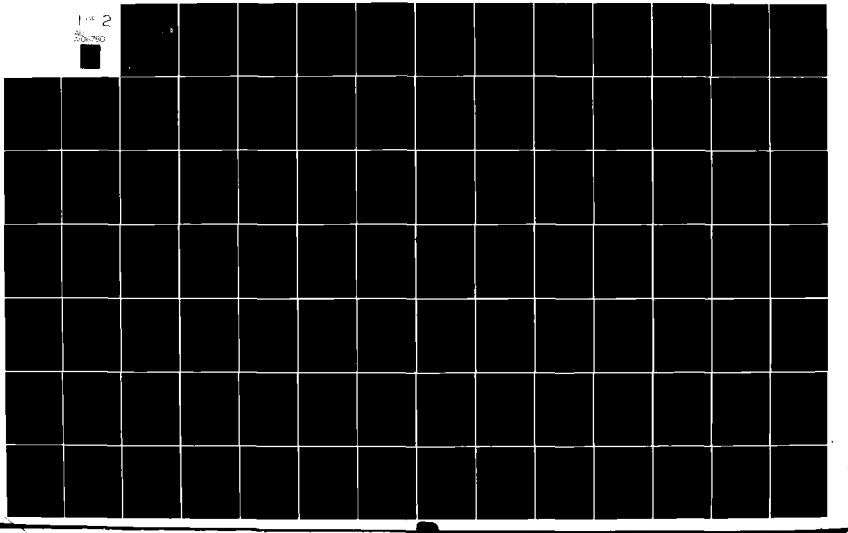

Final Report

Environmental Fate and Effects of N-phenyl-1-Naphthylamine and its Disposition and Metabolism in the Rat

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The photolysis of N-phenyl-1-naphthylamine (PNA) in aqueous solutions has been studied under natural sunlight as well as under laboratory conditions. Photodegradation of the material in sunlight is rapid and proceeds with a half-life of 5-8 minutes. The rate is, however, influenced by oxygen, and considerably enhanced rates are obtained in deoxygenated solutions. Photolysis in aerated solutions appears to lead to one primary product. This material is relative photostable, inert to sensitization by humic acid, and may also be pre-

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prepared by photolysis of PNA in nonaqueous solvents. Preliminary spectral data indicates that it may be the aniline adduct of 1,4-naphthoquinone.

PNA is degraded by microorganisms in lake water with a disappearance half-life of approximately 10 days. However, the presence of an external carbon and energy source, e.g. yeast extract, enhanced the microbial degradation of the chemical. The products of degradation included CO_2 (resulting from cleavage of the phenyl and/or naphthyl ring) and a dihydroxy derivative of PNA.

The acute toxicity of PNA to rainbow trout, bluegill sunfish and Daphnia magna was determined under static and flow-through exposure conditions. The 96-hour median lethal concentration (LC_{50}) ranged between 0.44 and 0.82 mg/ℓ for the two fish species. The flow-through LC_{50} values were not significantly different from the static LC_{50} values with the same species. The 8-day flow-through LC_{50} was 0.30 mg/ℓ for rainbow trout and 0.47 mg/ℓ for bluegill sunfish. The 48-hour LC_{50} for Daphnia magna ranged between 0.30 and 0.68 mg/ℓ.

The chronic toxicity of the PNA to Daphnia magna was determined under static-renewal exposure conditions. The 21-day LC_{50} was 0.06 mg/ℓ, which is about 1/10 of the mean 48-hour LC_{50} for this species. The highest non-lethal and lowest lethal concentrations in this test were 0.02 and 0.04 mg/ℓ, respectively.

The uptake and elimination of ^{14}C-PNA by bluegills was investigated in preliminary short-term static exposure experiments and in a definitive flow-through experiment. PNA was readily taken up by the fish in both experiments. Under flow-through conditions, equilibrium was reached apparently between 8 and 10 days. At this time, bioconcentration factors (based on PNA concentration) were 600, 339 and 2063 for whole fish, edible flesh and viscera, respectively. The uptake and depuration rate constants (whole fish) 14.7 hr^{-1}, and 0.14 hr^{-1}, respectively. Half of the [^{14}C]PNA-derived radioactivity in the fish was eliminated in about 2 days after transfer to clean flowing water. HPLC analysis of fish exposed to ^{14}C-PNA for 10 days indicated that about 50% of the radioactivity was present as PNA metabolite(s). A major metabolite in the fish extract was identified as a dihydroxy derivative of PNA.

The uptake and elimination of the ^{14}C-PNA by Daphnia magna was also investigated in a static test. Equilibrium was reached by 12 hours at which time the bioconcentration factor (based on total ^{14}C) was 637. Half of the [^{14}C]PNA-derived radioactivity in Daphnia was eliminated in about 2 days after transfer to clean water.

Preliminary results indicate that exposure to rainbow trout to sublethal concentrations of PNA may affect the feeding behavior of the fish.

The absorption, tissue distribution, excretion, and metabolism of PNA was studied in the male rat following oral administration. [^{14}C]PNA was well absorbed by the rat with themaximum plasma radioactivity levels being found within four hours after dosing. The radioactivity as well distribution in the tissues 24 hr after administration with the highest levels found in the fat, followed by liver, kidney and lung. The elimination of radioactivity from the plasma, liver, kidney and lung was biphasic showing an initial rapid decline followed by a second slower disappearance process. [^{14}]PNA-derived radioactivity was extensively excreted by rats, mainly via the feces. Approximately 35% of the administered dose was recovered in the urine and 60% in the feces of rats within 72 hr. The radioactivity excreted in the urine was primarily in the form of PNA metabolites. Incubation of PNA with rat-liver microsomes resulted in the formation of several metabolites. Two of the metabolites were identified as mono-and dihydroxy derivatives of PNA.

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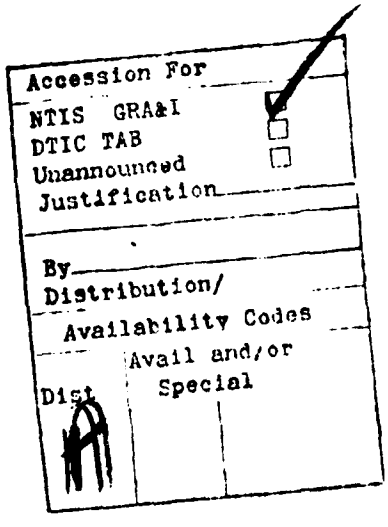


PART I. Environmental Fate and Effects of PNA

INTRODUCTION

N-phenyl-1-naphthylamine (hereafter referred to as PNA) is a lubricant additive widely used in Air Force operations. The chemical may be introduced into the aquatic environment as a result of accidental spills, waste discharge effluent from manufacturing plants, or during its use and disposal. The introduction of PNA into the aquatic environment is of great environmental concern because of its potential toxicity to aquatic fauna and flora and its possible adverse effects on man through his drinking-water supplies. Also, the chemical and its degradation products may accumulate in fish and could pose a health hazard if fish from contaminated waters were to be used as human food. Furthermore, PNA may be converted to other products as a result of biological or non-biological transformation in the aquatic environment. Since the transformation products may be more toxic and/or more persistent than the parent chemical, they may present additional pollution problems. Therefore, in order to fully evaluate the hazards associated with the release of PNA into the aquatic environment, it becomes important to study the environmental fate of the chemical sinces its persistence, disappearance or partial transformation will determine the degree of its hazardousness.

Several physical, chemical and biological factors determine the fate of a chemical in the aquatic environment. In order to have some information regarding the behavior of PNA in the aquatic environment, we have assessed the role of some of the processes which may determine the environmental fate of the chemical. This information will be useful in predicting the pathways of this chemical in the fresh-water environment and therefore in assessing the degree of both human and environmental exposure.

SPECIFIC OBJECTIVES

1. To study the photodegradation of PNA in an aqueous solution.
2. To study the biodegradation of PNA by aquatic micooraganisms.
3. To assess the tocixity of PNA to fish and Daphnia
4. To determine the uptake, elimination and metabolism of PNA in fish and Daphnia

MATERIALS AND METHODS

Chemicals

N-phenyl-1-napththylamine (PNA) was obtained from the Aldrich Chemical Company and converted to its hydrochloride salt. The conversion procedure involved treatment of the free base in methanol with concentrated HCl and recrystallization of the resulting solid material from methanol.

^{14}C-labeled PNA (N-phenyl-^{14}C(U) and naphthyl-1-^{14}C) with a specific activity of 5.68 mCi/mM was purchased from New England Nuclear, Boston, Mass. The radiochemical purity of this chemical was > 98%, determined by high-pressure liquid chromatography. Instagel, Permafluor V and Carbo-Sorb were purchased from Packard Instrument Co., Inc.

All PNA stock solutions were refrigerated and protected from light during storage.

Analytical Methods

The analytical methods used to determine PNA and its degradation products included high-pressure liquid chromatography (HPLC), gas liquid chromatography (GLC), mass spectrometry (MS), and radioassays.

High-Pressure Liquid Chromatography

Analyses were performed using a Waters Associates HPLC (Model 6000A) equipped with a reversed phase column (μ-Bondapak C_{18} or RP-2) and a Schoeffel SF 770 UV detector or Bioanalytical systems electrochemical detector. The column was eluted with a mixture of acetonitrile and water. Typical retention volume under a variety of conditions are shown in Table 1. The type of detector varied with the nature of the experiment. The electrochemical detector is far more sensitive than the UV detector (we estimate that < 0.1 ng can be quantified at an applied voltage of 0.7 v as compared to 10 ng for the UV detection at 215 nm) but is

specific to PNA only, and consequently, electrochemical detection was used where product analysis was not required. On the other hand, the UV detector was used in instances where detection of products was of importance.

Table 1

HPLC retention Volumes of PNA

Column	Mobile phase	Retn. volume (ml)
μC_{18} Bondapak	3:2 acetonitrile:water	15
RP-2	7:3 acetonitrile:0.1M acetate buffer[1]	4.6
RP-2	3:2 acetonitrile:0.1M acetate buffer	8.3
RP-2	1:1 acetonitrile:0.1M acetate buffer	15.8

[1]The buffer is necessary for electrochemical detection.

Gas-liquid Chromatography

GLC analysis was performed using a Hewlett Packard Model 8540 gas chromatograph equipped with a flame-ionization detector and a column containing 3% OV-1 on Chromosorb-W. The operating GLC conditions were as follows: 50 mℓ/min of N_2 carrier gas; injector, column and detector temperatures of 200°, 165°, and 325°, respectively.

Radioassays

All radioassays were performed using a Packard Tri-Carb Model 3255 liquid scintillation spectrometer equipped with automatic external standardization. All data were corrected for background interference, quenching, and counting efficiency. Aliquots of water and organic extracts were counted in Instagel. Samples of

tissues were oxidized by combusting in a Packard Sample Oxidizer Model 306 using 10 mℓ of the trapping solution Carbo-Sorb and 12 mℓ of the scintillation cocktail Permafluor (Packard Instrument Company). ^{14}C-PNA and metabolites eluting from the HPLC column were detected and quantified by a radioactivity flow detector (Radiometric Instrument and Chemical Company, Inc., Addison, IL.)

Photodegradation of PNA

The photodegradation of PNA in an aqueous solution was examined following irradiation with sunlight or light of 300 nm wavelength. The latter studies were conducted using a Rayonett Model RMR-400-mini photochemical reactor.

To characterize the products resulting from the photodegradation of PNA, the photolyzed solution was subjected to HPLC, and eluant fraction corresponding to the photodegradation product was collected. The eluate was extracted with ether and the extract was analyzed by combined GLC-mass spectrometer (Finnigan 3000 GS/MS/DS) at Cornell University's mas spectral facility, Ithaca, New York.

Degradation of PNA by Aquatic Microorganisms

These studies were done using samples of lake water and primary sewage effluent. Samples of water were obtained from the Oneida lake. Prior to starting the biodegradation studies, the microbial population in the water was determined using the standard serial dilution and plating techniques. The microbial population ranged from 1500 to 2200 colony forming units per mℓ. Sewage was collected from a local domestic waste-water treatment plant.

To determine the rate of biodegradation of PNA, the chemical was added to water in sterile Erlenmeyer flasks at a concentration of 2 ppm. Some of the water samples were supplemented with either nutrient broth or yeast extract (500 mg/mℓ). The flasks were stoppered with foam plugs and incubated on a rotary

shaker in the dark at 21 ± 1°C. Sterilized water samples containing PNA were included as controls to account for any loss of the chemical due to non-biological degradation. Two-mℓ aliquots of water were removed periodically, mixed with 2 mℓ of acetonitrile, filtered through a 0.2 µm teflon filter and analyzed for PNA by HPLC.

^{14}C-PNA was used to determine the products resulting from the biodegradation of the chemical. The evolution of $^{14}CO_2$ resulting from the microbial degradation of ^{14}C-PNA was measured using the biometer flask described by Bartha and Pramer (1). The water samples (50-ml) were incubated with 2 ppm of ^{14}C-PNA in the biometer flask and 0.1 N KOH was used as the CO_2-trapping solution in the side arm. The solution in the side arm was removed and replaced at appropriate intervals. The amount of ^{14}C in the CO_2-trapping solution was measured by liquid scintillation counting. The radioactivity collected in the KOH trap was verified as $^{14}CO_2$ by acidifying with HCl. At the termination of the experiment, the water samples were adjusted to pH 11 and extracted three times with diethyl ether. The aqueous phase was then adjusted to pH 2 and extracted three times with thyl ether. The ether extracts and the aqueous phase were assayed for radioactivity. The ether extract was dried under a stream of nitrogen, the residue was dissolved in acetonitrile and the acetonitrile extract was analyzed by HPLC using a Waters Associate high-pressure liquid chromatograph equipped with a UV detector and a radioactivity flow monitor.

In an attempt to obtain PNA degradation product(s) in quantities sufficient for spectral analysis, a separate large batch-culture experiment was conducted. PNA was added at a concentration of 2 ppm to 12 liters of lake water contained in 15-ℓ carboys. The water was supplemented with 500 ppm of nutrient broth. The carboys were incubated in the dark at 20°C; during the incubation, the cultures were continuously stirred and aerated. Aliquots of the water were

analyzed daily for PNA until 65% of the chemical had disappeared (10 days after incubation). The contents of carboy were adjusted to pH 12, and extracted three times with diethyl ether. The aqueous phase was then adjusted to pH 2 and extracted three times with ether. The combined ether extracts were concentrated to about 2 mℓ under vacuum and analyzed by combined GLC-mass spectrometery.

Toxicity of PNA to Aquatic Organism

Acute toxicity tests were conducted with bluegill sunfish (Lepomis macrochirus), rainbow trout (Salmo gairdneri) and water fleas (Daphnia magna) to assess the aquatic toxicity of PNA. In addition, a chronic toxicity test was conducted with D. magna. These freshwater species were chosen because they are representative, respectively, of warm water fishes, cold water fishes, and freshwater invertebrates and, as such, are recommended by the U.S. Environmental Protection Agency (2) for aquatic toxicity testing.

D. magna were obtained from a biological supply house and were cultured and tested in soft or hard reconstituted freshwater containing, respectively, 48 or 192 mg/ℓ $NaHCO_3$, 30 or 120 mg/ℓ $MgSO_4$, 30 or 120 mg/ℓ $CaSO_4.2H_2O$ and 2 or 8 mg/ℓ KCl. (pH = 7.2-7.6 or 7.6-8.0, hardness = 40-48 or 160-180 mg/ℓ as $CaCO_3$, alkalinity = 30-35 or 110-120 mg/ℓ as $CaCO_3$, respectively). These media are recommended by the U.S. Environmental Protection Agency (2) for culture and toxicity testing of freshwater invertebrates. The reagent grade water used to make this medium was deionized (>10 megohm-cm) and filtered through activated carbon and a 0.2 micron particle filter in a Barnstead NANOpure® water purification unit.

Stock D. magna cultures were maintained on a diet of baker's yeast, cerophyll, and algae (Chlorella pyrenoidosa) at 20 ± 2°C under a 16 hr light:8 hr dark photoperiod. Regular subculturing was carried out according to the method

of Dewey and Parker (3) in 4ℓ polyethylene separatory funnels equipped with nylon (1 mm mesh) screens to collect first instar (<24 hr old) young as they are produced from adult cultures.

Bluegill sunfish and rainbow trout were obtained from commercial fish hatcheries and maintained under flow-through conditions in round fiberglass tanks. The fish were maintained at 13 ± 2°C (rainbow trout) or 20 ± 2°C (bluegill sunfish) under a 16 hr light:8 hr dark photoperiod. The water supply used for fish maintenance and toxicity testing was Syracuse municipal water filtered and dechlorinated through activated charcoal. The characteristics of this water supply are given in Table 2.

Table 2

Characteristics of Filtered Municipal Water Supply

hardness, as $CaCO_3$	117.9 ± 10.4*	mg/l
alkalinity, as $CaCO_3$	89.1 ± 4.5	mg/l
calcium	91.4 ± 5.4	mg/l
chloride	5.9 ± 2.7	mg/l
total phosphate	0.15 ± 0.17	mg/l
total organic carbon	2.2 ± 1.7	mg/l
total suspended solids	<5	mg/l
conductivity	265 ± 17.3	µmhos/cm
iron	40 ± 15	µg/l
zinc	5	µg/l
copper	2.5	µg/l
ammonia	<10	µg/l
chlorine	<10	µg/l

*Number after ± represents 1 standard deviation.

Bluegill sunfish, rainbow trout, fathead minnows (Pimephales promelas) and channel catfish (Ictalurus punctatus) have been maintained on a diet of commercial trout feed for more than one year with less than 5% mortality using this water supply.

Fish Bioassays

Fish bioassays were conducted under static-renewal and flow-through exposure conditions. Test concentrations for static-renewal bioassays were prepared by adding purified PNA (as the hydrochloride salt) dissolved in ethanol to well-aerated test water. Equal amounts of ethanol wer added to each test container including the control. The ethanol concentration did not exceed 0.5 mℓ/ℓ water as recommended by the U.S. EPA (4). Test solutions were vigorously stirred with a glass rod and the test animals were then added (within 10 minutes). The solutions were not aerated during the tests. Before each definitive bioassay, preliminary range-finding bioassays were conducted to obtain an estimate of the LC_{50} (median lethal concentration). From this information, a series of concentrations was prepared based on a dilution factor of at least 0.6 from one concentration to the next lower concentration.

Bluegill sunfish (1.0 g mean weight) were exposed to five PNA concentrations, an ethanol control, and a control in 5 gallon wide-mouth jars that contained 15ℓ of well-aerated dechlorinated tap water. At the beginning of the test, ten fish were added at random to each jar to give a loading ratio of about 0.7 g fish/ℓ test solution. Mortalities were recorded daily for 8 days. Rainbow trout (0.6 g mean weight) were exposed to five PNA concentrations plus an ethanol control in 5 gallon wide-mouth glass jars that contained 15ℓ of tap water. Ten fish were added at random to each jar to give a loading ratio of about 0.4 g fish/ℓ test solution. Mortalities were recorded daily for four days. In both static-re-

newal tests, the fish were transferred daily to new test solutions and were not fed during the test. Dead fish were removed as soon as they were noticed. The pH and dissolved oxygen content of all test solutions were measured immediately after the beginning of the test and daily on the day-old solutions just after the fish were transferred to fresh solution.

Eight-day flow-through bioassays were conducted with bluegill sunfish and rainbow trout using a modified solenoid-operated proportional diluter (5). A stock solution of PNA dissolved in acetone was added to water with a syringe pump. The diluter delivered four toxicant concentrations (nominal 0.6 dilution factor) and control water (without acetone) to 30ℓ aquaria at 3ℓ/hr. This flow rate corresponds to 2.4 tank volumes per day and a 90% replacement time of about one day (4). At the beginning of each bioassay, ten fish were added at random to each of five aquaria. The mean weight of the fish was 1.8 g for bluegills and 4.4 g for rainbow trout, giving loading ratios of 0.6 g fish/ℓ, and 1.5 g fish/ℓ,respectively. The aquaria were aerated during the tests. The tests were conducted under a 16 hr light/8 hr dark photoperiod at temperatures of 20 $\pm$ 2°C with bluegills and 13 $\pm$ 2°C with trout. Mortalities were recorded daily and dead fish were removed as soon as they were noticed. The pH dissolved oxygen content of all test concentrations were measured at the beginning and end of the test. The fish were not fed until 4 days after beginning the test. They were then fed every other day until the end of the test.

The PNA concentrations during the flow-through bioassays were measured daily in each tank by the following procedure. One 100 mℓ water sample from each tank was extracted twice with 20 mℓ methylene chloride in a separatory funnel. The pooled extracts were evaporated to dryness under nitrogen gas and redissolved in 2 mℓ methanol. Two or more aliquots from each sample and methanol standards

were then analyzed by high pressure liquid chromatography (HPLC) using reverse phase columns (RP-2), 70:30 or 80:20 acetonitrile:water mixtures as the mobile phase and UV detection at 220 or 250 nm.

Daphnia Bioassays

Acute (48 hr) and chronic (21 day) toxicity tests were conducted with Daphnia under static or static-renewal exposure conditions.

Two static acute tests were conducted with soft water to determine the 48-hr LC_{50} of PNA for first instar (12 $\pm$ 12 hr old) and adult (7 $\pm$ 1 day old) daphnids. Test solutions were prepared by adding ethanol stock solutions of PNA to water to give five PNA concentrations (0.6 dilution factor) and equal concentrations of ethanol. For each test, groups of 10 daphnids were exposed in duplicate to the five PNA concentrations and two control treatments (with and without ethanol) in culture dishes containing 100 mℓ of treatment solution. The animals were not fed during the 48 hr exposure period. Mortalities were recorded at 24 and 48 hr.

Two other acute tests were conducted with first instar daphnids to determine the 48-hr LC_{50} of PNA in soft and hard water. Test solutions were prepared by adding ethanol stock solutions of PNA to soft or hard water to give six PNA concentrations (0.6 dilution factor) and equal concentrations of ethanol. For each test, groups of five daphnids were exposed in duplicate to the six PNA concentrations and two control treatments (with and without ethanol) in culture dishes continuing 100 mℓ of treatment solution. The animals were transferred to fresh solutions after 24 hr of exposure. Mortalities were recorded at 24 and 48 hr. Daphnids were not fed during the test.

A chronic static-renewal test was conducted in hard water to determine the LC_{50} of PNA over a 21-day exposure period. Test solutions were prepared

as previously described. Groups of five Daphnia (first instar) were exposed in triplicate to five PNA concentrations and two control treatments (with and without ethanol) in culture dishes containing 100 mℓ of treatment solution. Surviving animals were counted and transferred to fresh test solutions on Monday, Wednesday, and Friday during the 21-day test period. To minimize adsorption of PNA in fresh solutions glass, the dishes were filled with test solution at least 48 hr before being used. Just before transfer of the daphnids, old solutions were discarded and the dishes were filled with fresh solution. An aliquot of food suspension (yeast, cerophyll, trout starter feed) were added with mixing to each dish to give a food concentration of 30 mg/ℓ. The animals were then transferred from the old dishes.

Analysis of Toxicity Data

The results for all toxicity tests are expressed as LC_{50} and NOEC (no observed effect concentration) values in mg/ℓ. The LC_{50} is the concentration of the test substance which is lethal to 50% of the test organisms in a specified period of time. Lethality in fish is defined as the absence of gill movement and the lack of response to gentle prodding. Death of Daphnia was defined as lack of movement. The NOEC is the highest test concentration which had no lethal or sublethal effect.

Estimates of the LC_{50} values and their 95% confidence intervals were calculated by the moving average method of Thompson (6) when the data permitted. This method requires a constant dilution factor between the toxicant concentrations. If this method could not be used, the LC_{50} was estimated by the method of Litchfield and Wilcoxon (7) which requires at least two concentrations producing partial mortalities. Calculation of the 95% confidence interval of the LC_{50} by this method is possible only when at least one of the test concen-

trations produces a percent mortality between 16 and 84%. If the Litchfield and Wilcoxon method could not be used, the LC_{50} was estimated by graphical interpolation of a plot of log concentration versus percent mortality.

Uptake, Elimination, and Metabolism of PNA by Aquatic Organisms

Bluegill Sunfish

Preliminary experiments were conducted in which bluegills were exposed to ^{14}C-PNA under static exposure conditions. A stock solution of ^{14}C-PNA was prepared in methanol and added to four glass aquaria, each containing 15ℓ well-aerated dechlorinated tap water (20 ± 2°C), to give nominal PNA concentrations of 0.2 or 0.02 mg/ℓ. After thorough mixing of the test solutions, 15 bluegills (1.0 g average wt.) were added to each aquarium to give a loading ration of about 1 g fish/ℓ water. Triplicate 1 mℓ water aliquots and 1 or 2 fish were taken from each tank at 7, 24, and 49 hours after the start of the test. Water samples were counted for radioactivity by liquid scintillation counting. The fish were rinsed well, placed in plastic bags and killed by freezing. The frozen fish were dissected into head portion (including gills), viscera, and "edible flesh" (decapitated and eviscerated carcass). These tissues were placed into paper sample cups, dried and then weighed. Radioactivity in each sample was determined by burning the samples in a Packard Tri-Carb Sample Oxidizer and counting the radioactivity in the resulting $^{14}CO_2$ by liquid scintillation counting.

Another preliminary experiment was conducted to estimate the rate of PNA elimination. In this experiment bluegills were exposed to 0.2 mg/ℓ PNA for 48 hours, transferred to fresh 0.2 mg/ℓ PNA solution for an additional 72 hours, and then transferred to clean flowing water. The fish were sampled and counted as previously described at the end of the PNA exposure period and at 6, 24 and 168 hours after transfer to clean flowing water.

Following these preliminary experiments, a definitive experiment was conducted totally under flow-through exposure conditions. Water and stock solution of ^{14}C-PNA in acetone (specific activity = 4000 dpm/µg) were pumped to a mixing chamber where the solutions were thoroughly mixed by a magnetic stirrer before periodically siphoning into a 90ℓ glass aquarium fitted with a side drain. Water and ^{14}C-PNA stock solution were pumped, respectively, by a peristaltic pump or a syringe pump. The flow rates were checked daily and adjusted if necessary. The mean water flow rate to the aquarium was 8.8 ℓ/hr (range, 8.5-9 ℓ/hr), to give a 90% replacement time of about 24 hours (5).

After a stable concentration of ^{14}C-PNA was attained in the aquarium, 160 bluegill sunfish [(mean ± 1 S.D.) wet weight = 5.40 (± 1.88 g), range 2.3-11.5 g] were added to give initial loading factors of 9.5 g fish/ℓ water at any one time and 4.1 g fish/ℓ water passing through the aquarium in 24 hours. These loading factors decreased as fish samples were taken out during the exposure period. Triplicate 1 mℓ water aliquots were taken daily from the exposure tank and counted for radioactivity by liquid scintillation counting. Seven fish were sampled from the exposure tank at 1.5, 3, 6, 12, 24, 48, 96, 144, 192 and 240 hours after introducing the fish. Additional fish were sampled at 240 hours for determination of PNA residues. The remaining fish were then transferred to clean flowing water for the depuration phase. Seven fish were sampled at 3, 6, 12, 24, 52, 96, 192, 388.5, 576 and 768 hours after the start of the elimination phase. The test was carried out at 20 ± 2°C. The water was aerated and the fish were fed every other day.

The fish were killed in anesthetic (tricaine methanesulfonate), rinsed well in clean water, blotted dry, weighed and frozen. Each frozen fish was dissected into head portion (including gills), viscera, and "edible flesh" (decapitated and eviscerated carcass). The tissues were placed in a pre-weighed paper sample cups,

immediately weighed and allowed to dry. Radioactivity in each sample was determined by burning the samples in a Packard Tri-Carb Sample Oxidizer and counting the radioactivity in the collected $^{14}CO_2$ by liquid scintillation counting.

Some of the fish sampled at the end of the uptake phase were minced and homogenized in methanol with a Polytron homogenizer. The slurry was stirred for five minutes and centrifuged at 12,000 rpm for five minutes. The residue was resuspended in about 50 mℓ methanol, stirred for 30 minutes and centrifuged as before. This procedure was repeated for a third extraction. The pooled extracts were concentrated under vacuum and the extract was then filtered through a 0.45 μ teflon Millipore filter. The radioactivity in the filtered extract was quantified by liquid scintillation counting. The extract was subjected to HPLC using a Waters Associates liquid chromatograph equipped with UV detector and a Flo-One radioactivity detector. Analysis by HPLC was performed on a Waters μ-Bondapak C_{18} column which was eluted with acetonitrile:water (70:30). The methanol extract was also subjected to analysis by combined GLC-MS.

Daphnia magna

The uptake and elimination of ^{14}C-PNA by D. magna was measured under static exposure conditions in hard reconstituted fresh water. To prepare the exposure solution, ^{14}C-PNA (specific activity = 4.67×10^4 dpm/μg) was dissolved in acetone and then added with mixing to 1ℓ water. This solution was then added with mixing to 9ℓ water containing adult D. magna to give a final nominal concentration of 40 μg/ℓ PNA (∿ 1/10 of 48 hr LC_{50}). The D. magna had been transferred to the exposure container (5 gal-glass jar) from a fed stock culture in the same water 24 hr before adding the PNA solution. Triplicate 1 mℓ water samples were collected immediately after PNA addition and after 3, 6, 12, 24, 36, 48 and 72 hrs, and counted for radioactivity in 15 mℓ Instagel$^{(R)}$ by liquid scintillation counting.

D. magna samples were collected by net at the same time intervals starting at 3 hr of exposure. After 72 hr of PNA exposure, the remaining D. magna were collected by net, rinsed by dipping the net in three changes of clean water and added to 10ℓ of uncontaminated water which contained a freshly added suspension of 30 mg/ℓ yeast as food. Triplicate 1 mℓ water samples and D. magna samples were collected at 24 and 53 hr after beginning the elimination phase. All D. magna samples were dipped in three changes of clean water and then added to about 500 mℓ clean water. The animals were then collected on a cellulose filter using a Millipore filter apparatus. The animals were then carefully transferred by spatula to two pre-weighed paper sample cups, which were immediately weighed to determine wet weight. The samples were air-dried to a constant measured dry weight. Radioactivity in each sample was determined by burning the samples in a Packard Tri-Carb Sample Oxidizer and counting the radioactivity in the collected $^{14}CO_2$ by liquid scintillation counting. In order to obtain additional information concerning the elimination of ^{14}C-PNA by D. magna, this experiment was repeated using the same sampling techniques. D. magna were exposed to 40 µg/ℓ ^{14}C-PNA for 48 hours in 15ℓ of hard water. Triplicate water samples were collected at the start and after 24 and 48 hours during the uptake phase. A D. magna sample was taken at 48 hours and the remaining animals were transferred to clean water. D. magna samples were obtained after 24, 48, 96 and 192 hours in clean water. The sampling and ^{14}C counting procedures were the same as previously described.

RESULTS

Absorption Spectrum of PNA

The limited solubility of PNA in water precludes accurate spectral measurement in aqueous solutions, and consequently, the spectrum was obtained in methanol. A sample of PNA (as the hydrochloride) was purified by two recrystallizations from methanol and dried under vacuum in the dark. A methanolic solution of this material showed two peaks at 338 nm (ε = 8,580) and 252 nm (ε = 17,400) in addition to end absorption.

PHOTODEGRADATION OF PNA

In view of extensive conjugation present in PNA, it seemed likely that the compound would absorb sunlight and undergo photodegradation. Accordingly, a number of experiments were conducted to determine the rate of environmental photolysis of PNA, the nature of the products and the conditions which govern reactivity.

Rate of Degradation of PNA in Sunlight

Five-mℓ aliquots of an aqueous solution of PNA, in cylindrical quartz tubes (1.3 cm ID) were sealed with parafilm to prevent evaporation, and exposed to sunlight. Tubes were withdrawn periodically and solutions were analyzed in duplicate by HPLC. The results of two sets of experiments presented in Table 3 yield rate constants of 0.0083 min^{-1} and 0.121 min^{-1} which correspond to half-lives of 8.4 min and 5.7 min, respectively. The latter result is probably more reliable, since a wider range of measurements was made. In any event, it is clear that photolysis is an environmentally important route for the degradation of the chemical in the aquatic environment.

Table 3

Degration of PNA in Sunlight

Date	Min of Photolysis	PNA (ppb)	Percent Degradation
5/7/79	0	1350	
11:30 a.m.	10	392	71.0
	21	147	89.1
	31	76.4	94.3
	46	27.3	98.0
6/6/79	0	1160	
11:30 a.m.	2	1070	7.75
	4	751	35.3
	6	481	58.5
	8	384	66.9
	11	293	74.7
	15	193	83.4
	20	110	91

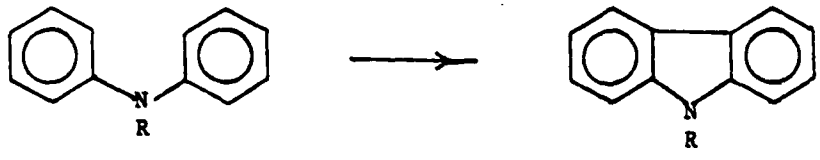
Influence of Oxygen on the Rate of Photolysis of PNA

Oxygen can frequently alter the rate of photolysis of a compound by interfering with the reaction sequence. For example, if the reaction proceeds via radicals, oxygen quenching may occur. Furthermore, oxygen can deactivate the excited state, particularly if it is a triplet. We determined the effect of oxygen on the rate of photolysis of PNA in order to obtain some insight into the mechanism of the reaction. a 1.38 ppm solution of PNA was prepared in water, and 10 mℓ aliquots were transferred to two identical quartz tubes. One of the tubes was degassed by two freeze-pump-thaw cycles. The two tubes were photolyzed together in a Rayonet RMR-400 merry-go-round reactor for 4.1 min. at 300 nm and analyzed by HPLC. The results presented below show that degradation proceeds to a much greater extent in the deoxygenated medium

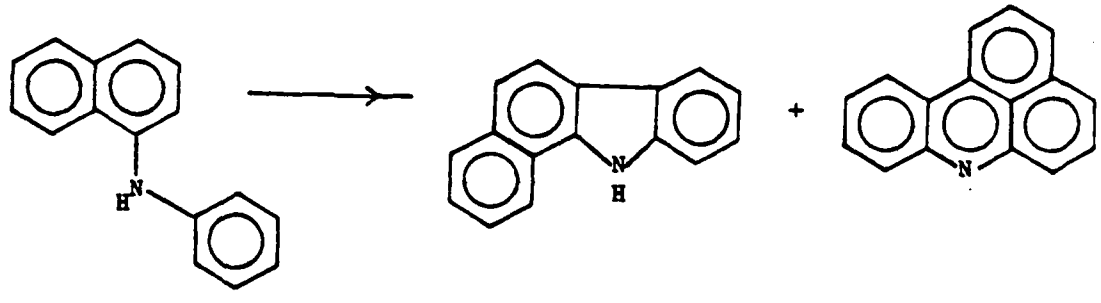
	PNA (ppm)
Before photolysis	1.38
After photolysis	0.85
Degassed, after photolysis	0.054

Products of PNA Photodegradation

Although little is know about the photochemistry of PNA, the photoproducts of diphenylamines have been characterized (8, 9). For example, diphenylamine and its N-substituted derivatives form carbazoles upon oxidation in hexane as shown below. If a similar process were to occur with PNA, the products would



include benzcarbazoles or benzacridines, both of which are almost certainly mutagenic and/or carcinogenic. Hence, dureing our analysis of PNA photoproducts, particular attention was paid to the possibility of benzcarbazole and benzacridine formation.



A number of preliminary experiments were conducted in order to determine the general nature of the photoproducts. We observed that HPLC analysis of photolyzed solutions of PNA showed several product peaks, one of which predominated. This major product has an absorption maxima at approximately 260 nm as determined by

"scanning" the peak with the variable wavelength UV detector. A typical scan of a 1.3 ppm PNA solution photolyzed at 300 nm for 20 min is illustrated in Fig. 1. Ether extraction of the photolysate and GC analysis showed one major peak in addition to residual PNA. This peak also appears when the fraction corresponding to the major HPLC product peak is collected, worked up and analyzed by GC. Mass spectra of this peak were obtained with a GC-MS-DS instrument, and electron impact and chemical ionization spectra are illustrated in Figs. 2 and 3, respectively.

In order to quantify the extent of conversion of PNA to this product, an experiment was conducted with ^{14}C-PNA. A 10 mℓ aqueous solution of this material was photolyzed at 300 nm with samples being withdrawn at 12 and 35 min. These samples were analyzed by HPLC, and the fractions corresponding to the major product and to PNA were collected and counted for radioactivity. The results listed in Table 4 clearly illustrate that the product determined by GC and HPLC

Table 4

Photolysis of ^{14}C-PNA

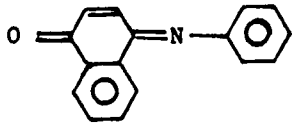
Min. of Irradiation	PNA (ppm)	PNA (dpm)[1]	Product (dpm)[1]	Percent conversion of PNA to product
0	0.767 ± 0.5%	2667 ± 0.4%	0	
12	0.521 ± 1.2%	1929 ± 2.0%	580 ± 1.4%	79
35	0.259 ± 3.6%	1064 ± 0.3%	1305 ± 3.1%	81

[1]Analysis of fractions collected from the HPLC. Initial injection volume was 100 µl.

is indeed the major product. Furthermore, assuming that the HPLC peak shapes of PNA and the product are comparable, then the extinction coefficient of the product at 260 nm (the analytical wavelength) is 1.88 times that of PNA at 260 nm. Finally, the product appears to be photostable relative to PNA, since the percent conversion of PNA to product remains constant at approximately 80% after 12 min of photolysis.

The product may also be generated at much higher concentrations by conducting the photolysis in methanol or cyclohexane. For example, photolysis of a 1250 ppm solution of a PNA in methanol for 17 hours at 330 nm. in the merry-go-round apparatus gave a brown solution. The solution was concentrated to approximately 2 ml. and analyzed by HPLC. The fraction corresponding to the product peak referred to in Table 4 was collected and analyzed by uv. spectrophotometry. Two bands at 255 nm. and 333 nm. were observed. Ether extraction of the collected fraction and GC analysis of the ether concentrate indicated that the product was identical to that formed during photolysis of PNA in water.

The above experiments do not allow us to identify the product, but it permits tentative appraisal of its structural features. The absorption spectrum of the product does not appear to differ greatly from that of PNA, and it is likely that the product incorporates the basic phenylnaphthylamine skeleton. A structural modification appears to have occurred at nitrogen, since the product is polarographically inactive under conditions where PNA is active, which implies that the oxidation potential is considerably higher than that of PNA. The mass spectra of the product are not definitive, but are consistent with a compound of molecular weight 233. In our view, the most likely structure for the product is the Schiff base shown below.



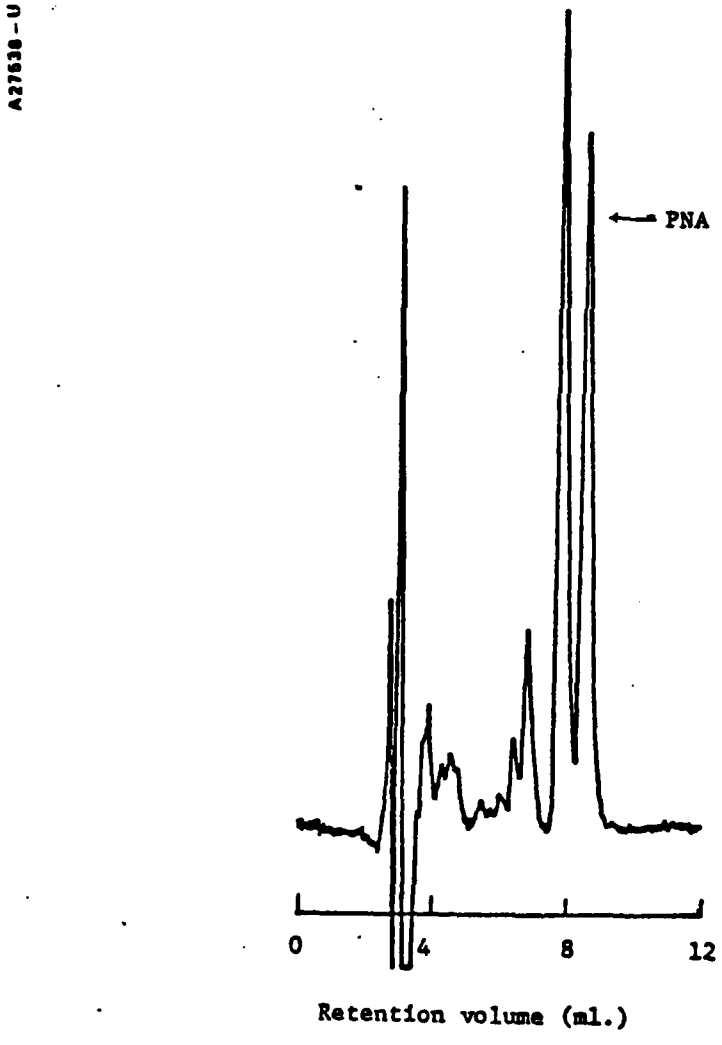


Figure 1. HPLC scan of PNA photolysate

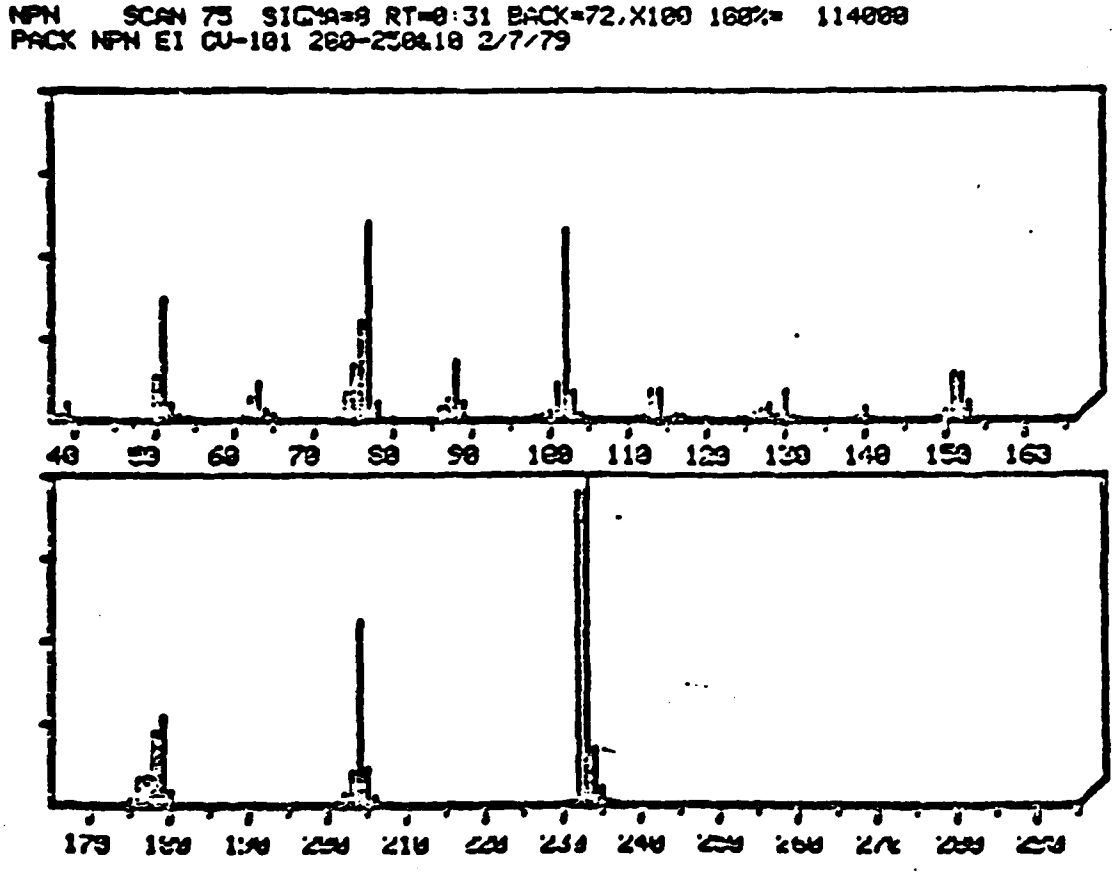


Figure 2. Electron Impact mass spectrum of PNA Photoproduct

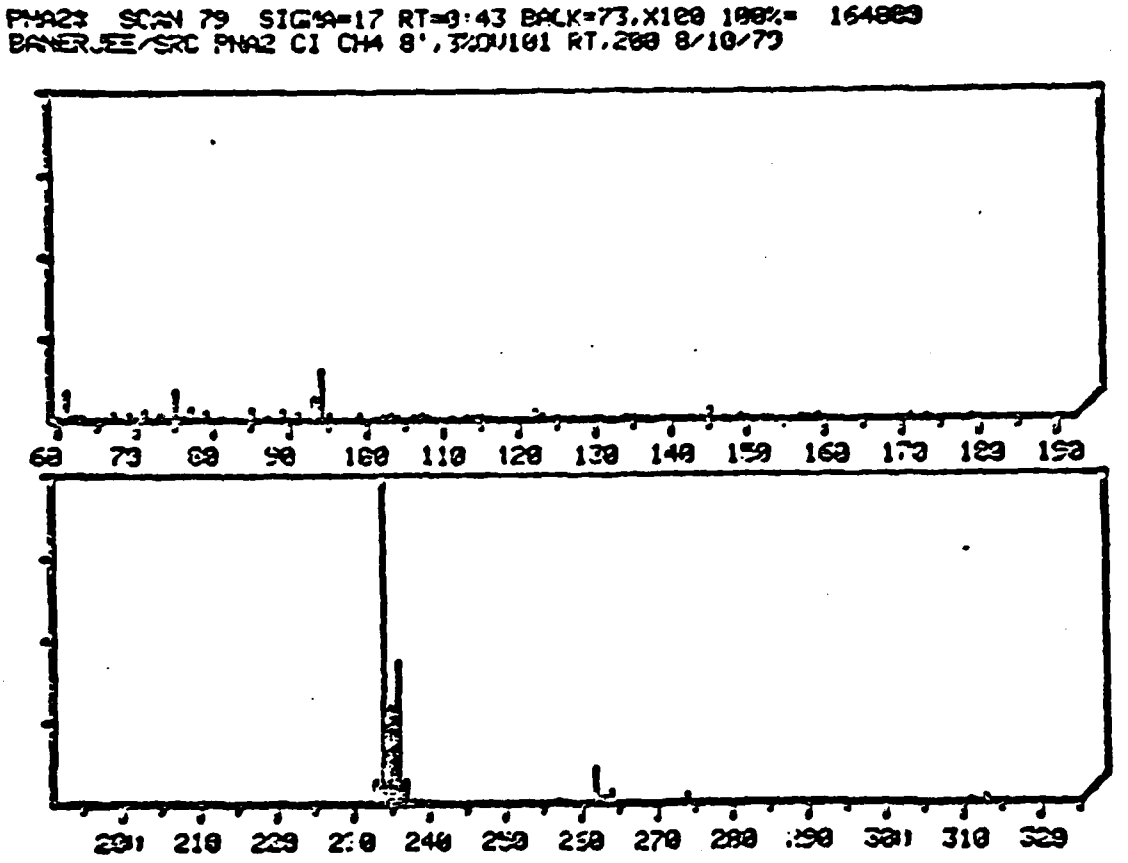


Figure 3. Chemical ionization mass spectrum of PNA photoproduct

Degradation of PNA Photoproduct

Since the photodegradation of PNA is rapid and is almost certainly of environmental importance, a brief study was initiated to determine the fate of the photoproduct referred to in the previous section. The photoproduct was collected by HPLC from a solution obtained by photolysis of PNA, and photolyzed in turn at 3000 Å for 24 hrs. Approximately 30% degradation was found to have occurred. Under similar conditions, the degradation of PNA is virtually complete in one hour. An attempt to sensitize the degradation of the photoproduct was made by photolyzing the PNA photoproduct in the presence of 1.9 ppm humic acid. However, very little degradation occurred after 5 hours of photolysis. These results suggest that the photoproduct from PNA resists fruther photodegradation.

STABILITY OF PNA IN WATER

Environmental oxidation or hydrolysis can play a major role in the degradation of a chemical, and in order to assess the importance of one or both of these factors, the stability of PNA in dilute aqueous solution was examined. In a typical experiment, 5 mℓ of an aqueous solution of PNA was maintained in the dark in 2 glass tubes of head space 24 mℓ, and were analyzed periodically by HPLC. The results, presented in Tables 5 and 6 suggest that PNA is removed from solution, but that this removal levels off with time. Behavior of this type is inconsistent with either oxidative or hydrolytic processes, particularly since no degradation products could be detected and we reasoned that adsorption to the glass surface could account for the observed decrease in concentration. Accordingly, we set up a similar experiment with ^{14}C-PNA, where any disappearance of radioactivity from the solution could only be accounted for by adsorption, and from aqueous solutions, and consequently oxidation and hydrolysis are either unimportant or of very limited importance from an environmental viewpoint.

Table 5

Stability of PNA in Water

Hours	PNA (ppb)	
	Tube 1	Tube 2
0	582	582
142	416	390
309	365	338
477	328	292
669	299	272
813	302	260

Table 6

Stability of PNA in Water

Hours	PNA (ppb)[1]	
	Tube 1	Tube 2
0	871	730
24	760	730
48	780	720
120	750	690
360	690	620
576	680	570

[1]Obtained by liquid scintillation counting and is consequently a measure of total radioactivity in solution.

BIODEGRADATION OF PNA BY AQUATIC MICROORGANISM

Rate of Biodegradation

In the sewage samples unsupplemented with nutrient broth or yeast extract, about 50% of the originally added PNA had disappeared within five days (Figure 4). Assuming that the degradation followed the first-order kinetics, the rate constant and half-life of degradation were 6.8 x $10^{-3}hr^{-}1$ and 4.2 days respectively. Degradation of the chemical continued thereafter at a slowere rate and only 3% of the original chemical remained after 18 days. Less than 20% of the initially added PNA had disappeared from sterile sewage after 18 days. Therefore, the decrease in the amount of PNA in the non-sterile sewage is attributed to the action of microorganisms in sewage. The addition of yeast extract or nutrient broth the sewage enhanced the biodegradation of PNA. In the supplemented sewage, greater than 75% of the orginally added chemical had disappeared after 2 days ($t_{1/2}$ = 1.2 days) and no PNA could be detected after 18 days. Thus, although PNA was readily degraded in unsupplemented sewage, the addition of an external carbon and energy source enhanced the rate of degradation (k = 2.4 x $10^{-2}hr^{-1}$).

The degradation of PNA in Oneida lake water was slower than that noticed in the sewage effluent (Figure 5). Nearly 50% of the chemical had degraded within 10 days. Subsequently, there was no reduction in the amount of PNA in the water. The addition of nutrient broth to the lake water enhanced the biodegradation of PNA. In the water samples containing nutrient broth, the degradation continued with time and less than 10% of the chemical was detected in the water after 18 days. Under these conditions, the rate constant and half-life for degradation were 7.9 x $10^{-3}hr^{-1}$ and 7.2 days, respectively.

Products of Biodegradation

The degradation of ^{14}C-PNA was also determined by measuring the evolution of $^{14}CO_2$ from the water samples treated with ^{14}C-PNA. The evolution of $^{14}CO_2$

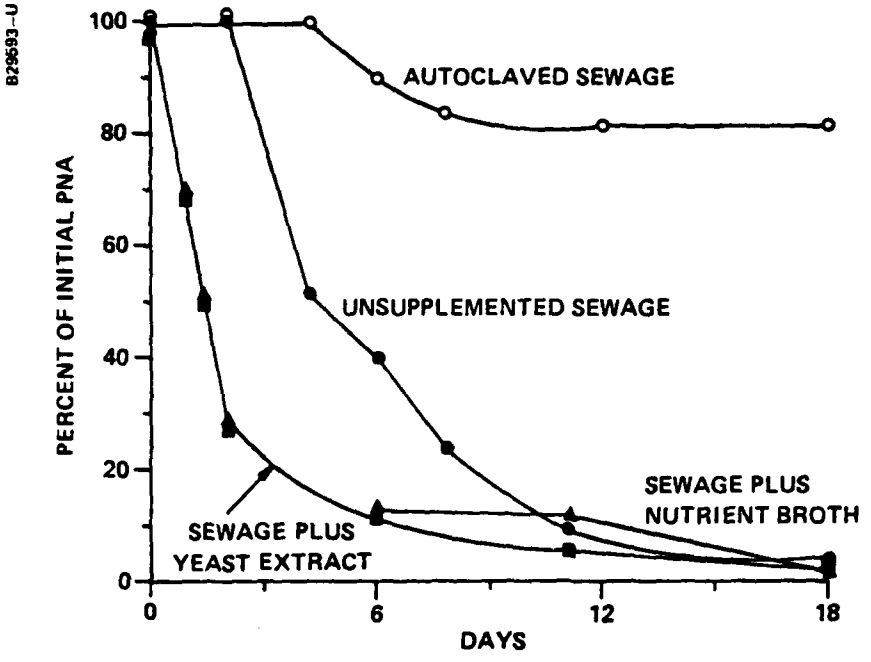


Figure 4. Degradation of PNA in sewage effluent incubated with 2 ppm of PNA.

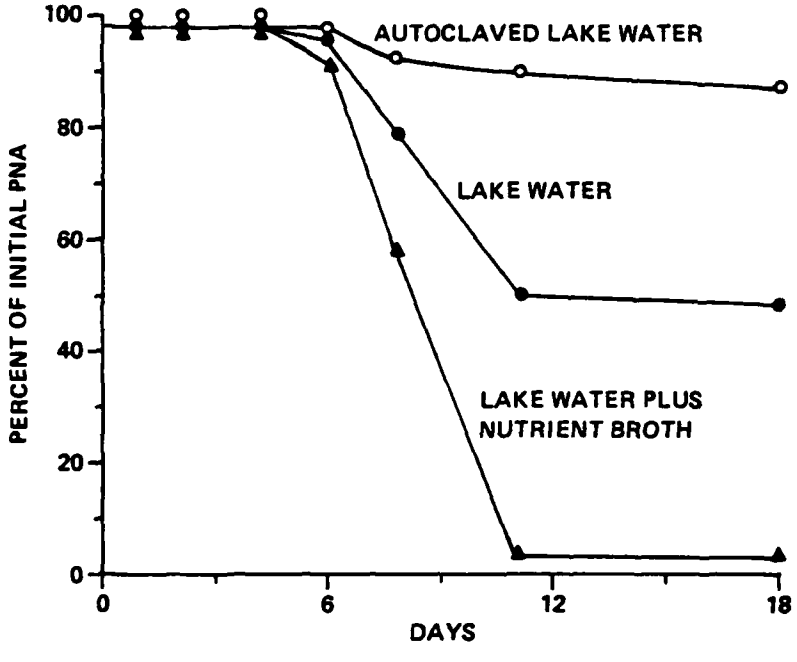


Figure 5. Degradation of PNA in lake water incubated with 2 ppm of PNA.

from the sewage effluent and Oneida lake water treated with 2 ppm of ^{14}C-PNA is shown in Figures 6 and 7. In the sewage, evolution of $^{14}CO_2$ was evident 6 days after incubation. About 13% of the initial ^{14}C added was evolved as $^{14}CO_2$ within 15 days of treatment. Thereafter, the evolution of $^{14}CO_2$ continued at a slower rate and amounted to 21% of the initial ^{14}C after 35 days. The presence of nutrient broth enhanced the rate of $^{14}CO_2$ evolution so that 27% of the initial ^{14}C was recovered as $^{14}CO_2$ after 35 days. $^{14}CO_2$ evolution was alos noticed in the Oneida lake water samples although both the rate and extent of ^{14}C-PNA degradation was considerably lower than that noticed in the sewage samples. As in sewage samples, addition of nutrient broth increased the rate of biodegradation of the ^{14}C-PNA to $^{14}CO_2$.

HPLC analysis of the ether extracts of the lake water incubated with ^{14}C-PNA for 10 days showed the presence of a major metabolite beside the parent compound (Figure 8). The metabolite(s) with a retention volume of 6 mℓ contained about 95% of the extractable radioactivity while about 5% of the radioactivity was present as unmetabolized PNA.

To characterize the product(s) resulting from the microbial degradation of PNA, a large batch volume of Oneida lake water was incubated with PNA and the water was analyzed after 10 days. A diethyl ether extract of the water contained two metabolites which are tentatively identified by GLC-MS of the extract as dihydroxy derivative of PNA (m/e 252) and N-acetyl PNA (m/e 262) (Figures 9 and 10). In the case of dihydroxy derivative of PNA, the positions of the hydroxyl groups in the molecule cannot be assigned on the basis of mass spectrum alone.

The results provide evidence that PNA undergoes hydroxylation and ring cleavage. Since PNA was labeled with ^{14}C both in the phenyl ring and at position position 1 of the naphthyl ring, evolution of $^{14}CO_2$ indicates cleavage of

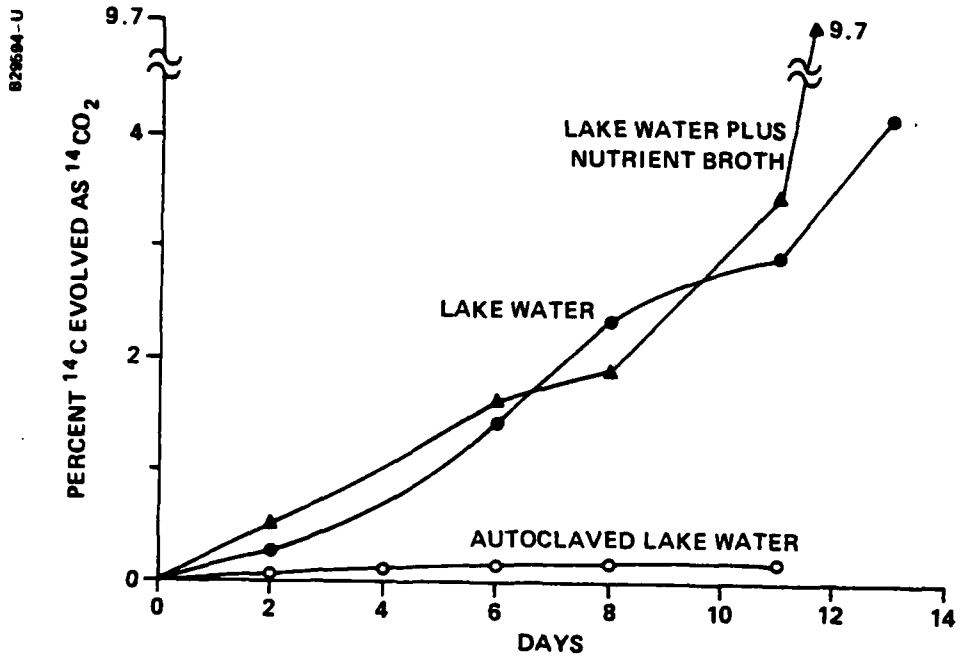


Figure 6. $^{14}CO_2$ evolution from lake water incubated with 2 ppm of ^{14}C-PNA.

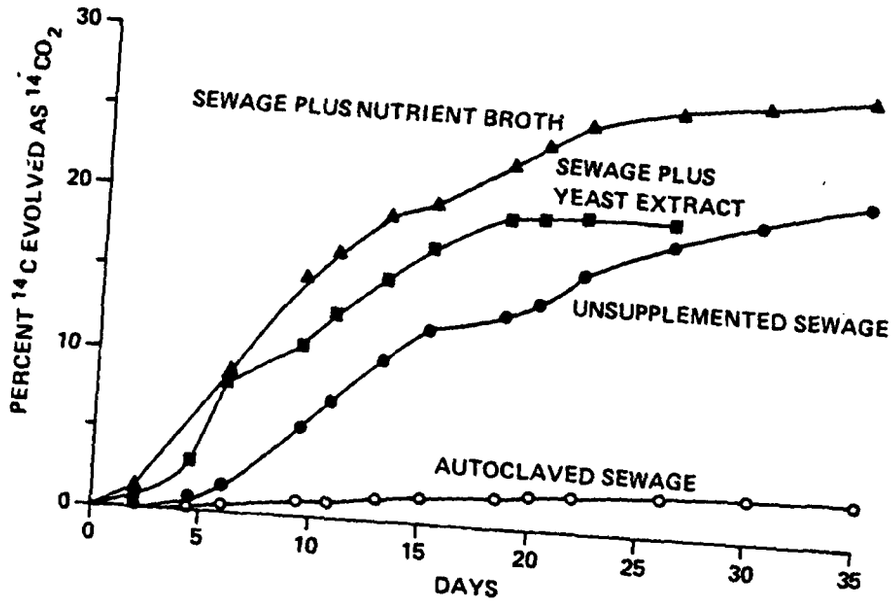


Figure 7. $^{14}CO_2$ evolution from sewage effluent incubated with 2 ppm of ^{14}C-PNA.

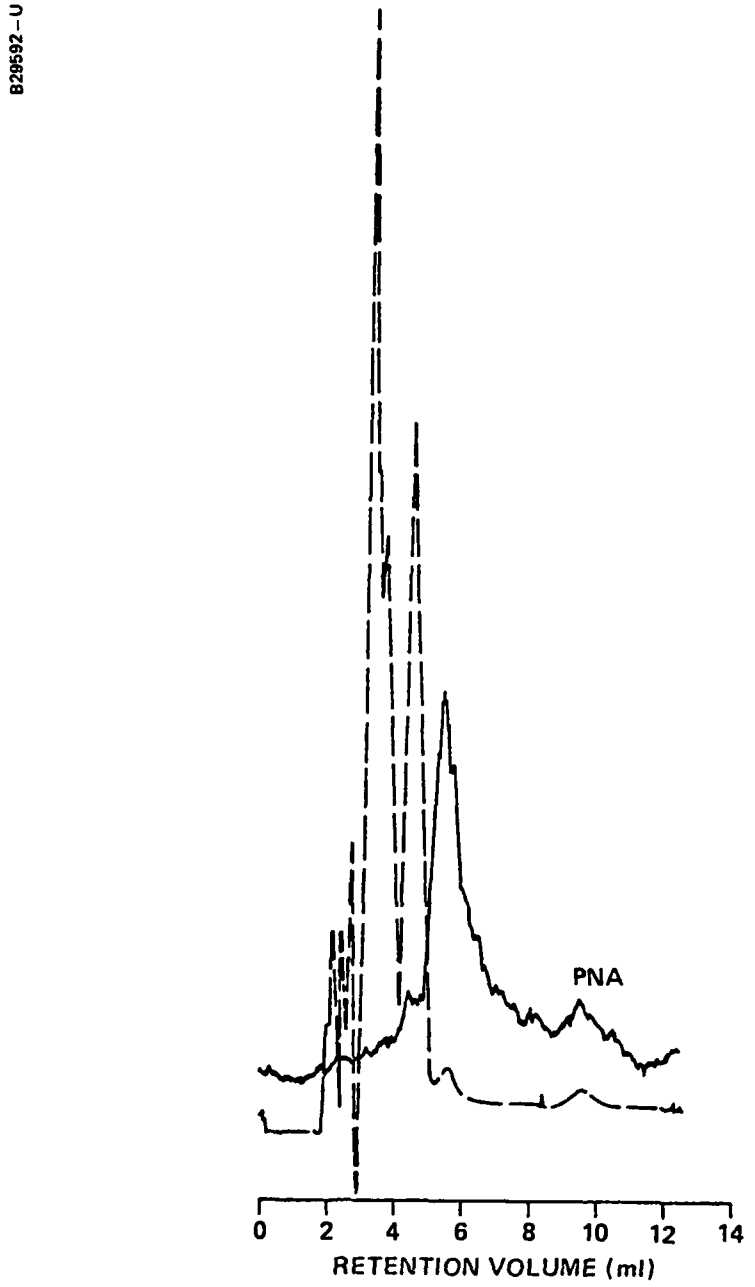


Figure 8. HPLC Profile of the Ether Extract of Lake Water Incubated with ^{14}C-PNA. UV Detector (---); Radioactivity Flow Detector (-).

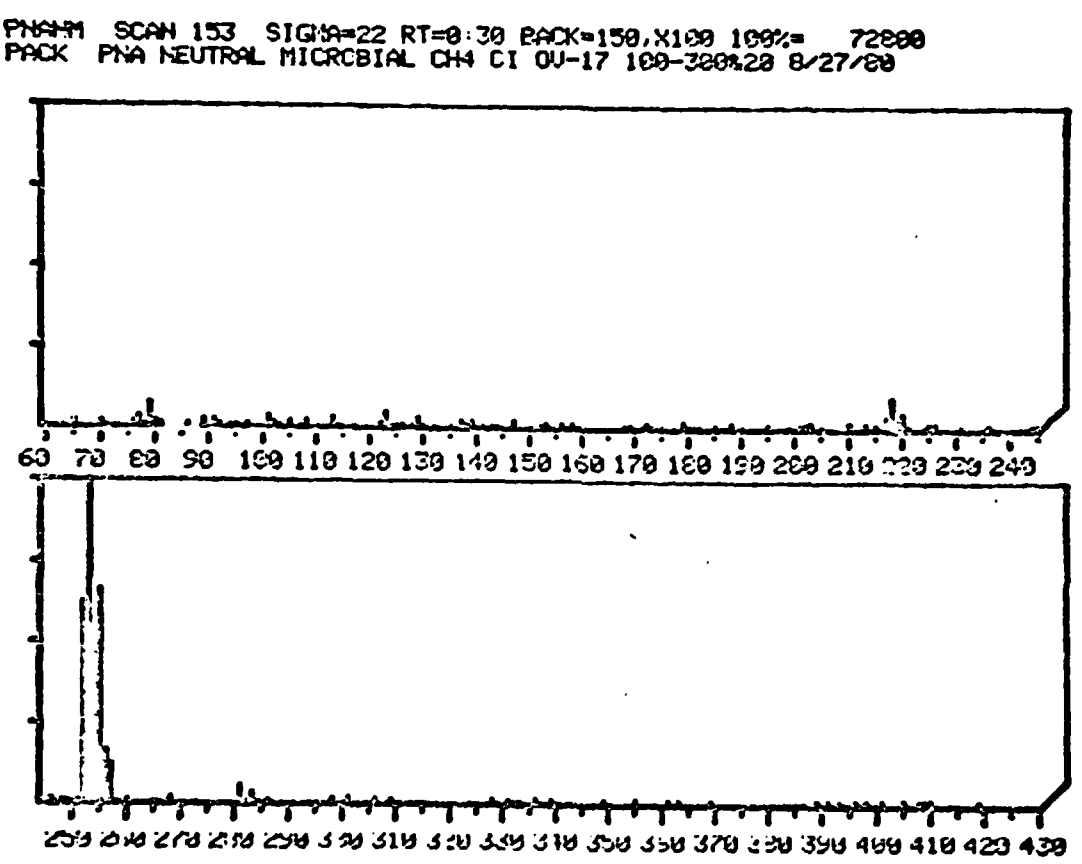


Figure 9. Mass spectrum of dihydroxy PNA and (hydroxy imino quinone ?) formed during the metabolism of PNA by aquatic microorganisma.

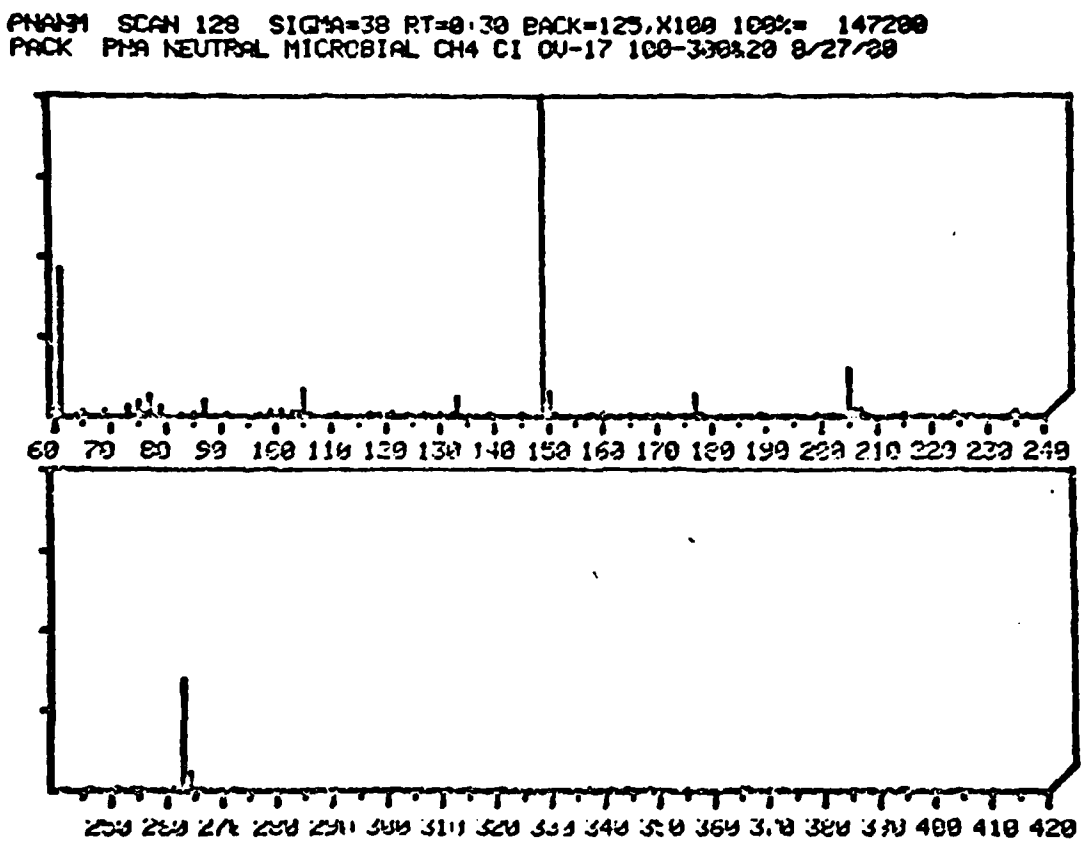


Figure 10. Mass spectrum of N-acetyl PNA formed during the metabolism of PNA by aquatic microorganisms.

the phenyl moiety, and/or cleavage at the naphthyl-1-^{14}C position. As the naphthalene ring is more susceptible to oxidation than the phenyl ring, we speculate that PNA metabolism by the microorganisms involves hydroxylation at a position ortho to the amino group followed by ring cleavage at this position. Similar metabolic pathways for the hydroxylation and subsequent ring opening of naphthalene have been proposed by Davies and Evans (10).

The results of this study demonstrate that PNA is extensively degraded to the extent of ring cleavage by the action of aquatic microorganisms. Addition of an external carbon and energy source (yeast extract, nutrient broth) appears to significantly increase the rate of biodegradation of PNA. The addition of yeast extract or nutrient broth may enhance the degradation of PNA by (i) increasing the number of microorganisms capable of degrading the chemical or (ii) serving as carbon or energy source for microorganisms capable of co-metabolizing PNA.

TOXICITY OF PNA TO AQUATIC ORGANISMS

The mortality data for the acute toxicity tests with D. magna, bluegill sunfish and rainbow trout are presented in Table 7-14. The mortality data for the chronic test with D. magna are presented in Table 15. The LC_{50} and NOEC values are given in Table 16.

Table 7

Cumulative Mortality Data for Daphnia magna (1st instar) in Soft Water During Static Acute Exposure

Nominal[a] Concentration (mg/ℓ)	0 hr.		24 hr.		48 hr.	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
1.00	0/20	0	10/20	50	18/20	90
0.60	0/10	0	0/10	0	10/10	100
0.36	0/20	0	0/20	0	11/20	55
0.22	0/20	0	0/20	0	6/20	30
0.13	0/20	0	0/20	0	1/20	5
Ethanol Control	0/20	0	1/20	5	3/20	15
Control	0/20	0	0/20	0	1/20	5

[a]Each PNA concentration and the ethanol control contained 0.5 mℓ ethanol/ℓ water.

Table 8

Cumulative Mortality Data for Daphnia magna (adults) in Soft Water During Static Acute Exposure

Nominal[a] Concentration (mg/ℓ)	0 hr.		24 hr.		48 hr.	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
1.00	0/17	0	0/17	0	11/17	65
0.60	0/20	0	1/20	5	10/20	50
0.36	0/20	0	0/20	0	1/20	5
0.22	0/20	0	2/20	10	4/20	20
0.13	0/20	0	1/20	5	1/20	5
Ethanol Control	0/20	0	0/20	0	1/20	5
Control	0/20	0	0/20	0	2/20	10

[a]Each PNA concentration and the ethanol control contained 0.33 mℓ ethanol/ℓ water.

Table 9

Cumulative Mortality Data for Daphnia magna (1st instar) in Soft Water During Static-Renewal Exposure

Nominal[a] Concentration (mg/ℓ)	0 hr.		24 hr.		48 hr.	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
1.00	0/10	0	7/10	70	8/10	80
0.60	0/10	0	3/10	30	4/10	40
0.36	0/10	0	1/10	10	1/10	10
0.22	0/10	0	0/10	0	0/10	0
0.13	0/10	0	0/10	0	0/10	0
0.08	0/10	0	0/10	0	0/10	0
Ethanol Control	0/10	0	1/10	10	1/10	10
Control	0/10	0	0/10	0	0/10	0

[a]Each PNA concentration and ethanol control contained 0.2 mℓ ethanol/ℓ water.

Table 10

Cumulative Mortality Data for Daphnia magna (1st instar) in Hard Water During Static-Renewal Exposure

Nominal[a] Concentration (mg/ℓ)	0 hr.		24 hr.		48 hr.	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
1.00	0/10	0	7/10	70	8/10	80
0.60	0/10	0	3/10	30	4/10	40
0.36	0/10	0	0/10	0	0/10	0
0.22	0/10	0	0/10	0	0/10	0
0.13	0/10	0	0/10	0	0/10	0
0.08	0/10	0	0/10	0	0/10	0
Ethanol Control	0/10	0	0/10	0	1/10	10
Control	0/10	0	0/10	0	0/10	0

[a]Each PNA concentration and ethanol control contained 0.2 mℓ ethanol/ℓ water.

Table 11

Cumulative Mortality Data for Bluegill Sunfish Tested under Static-Renewal Exposure Conditions

Nominal[a] Concentration (mg/ℓ)	0 hr.		48 hr.		96 hr.		144 hr.		192 hr.	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
1.00	0/9	0	0/9	0	7/9	78	9/9	100	9/9	100
0.60	0/10	0	0/10	0	1/10	10	4/10	40	8/10	80
0.36	0/10	0	3/10	30	3/10	30	3/10	30	3/10	30
0.22	0/9	0	1/9	11	2/9	22	2/9	22	2/9	22
0.13	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
Ethanol Control	0/9	0	0/9	0	0/9	0	0/9	0	0/9	0
Control	0/9	0	0/9	0	0/9	0	0/9	0	0/9	0

[a]Each PNA concentration and the ethanol control contained 0.33 mℓ ethanol/ℓ water.

Table 12

Cumulative Mortality Data for Rainbow Trout Test Under Static-Renewal Exposure Conditions

Nominal[a] Concentration (mg/ℓ)	0 hr.		24 hr.		48 hr.		96 hr.	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
1.00	0/10	0	2/10	20	7/10	70	10/10	100
0.60	0/10	0	0/10	0	10/10	100	10/10	100
0.36	0/10	0	0/10	0	0/10	0	1/10	10
0.22	0/10	0	0/10	0	0/10	0	0/10	0
0.13	0/10	0	0/10	0	0/10	0	0/10	0
Ethanol Control	0/10	0	0/10	0	0/10	0	0/10	0

[a]Each PNA concentration and the control contained 0.067 mℓ ethanol/ℓ water.

Table 13

Cumulative Mortality Data for Bluegill Sunfish Tested Under Flow-Through Exposure Conditions

Nominal[a] Concentration (mg/ℓ)	0 hr.		48 hr.		96 hr.		144 hr.		192 hr.	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
Test 1										
0.53	0/10	0	0/10	0	0/10	0	6/10	60	9/10	90
0.35	0/10	0	0/10	0	0/10	0	1/10	10	1/10	10
0.18	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
0.11	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
Control	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
Test 2										
1.05	0/10	0	4/10	40	9/10	90	10/10	100	10/10	100
0.64	0/10	0	0/10	0	1/10	10	9/10	90	10/10	100
0.38	0/10	0	0/10	0	0/10	0	0/10	0	1/10	10
0.24	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
Control	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0

Table 14

Cumulative Mortality Data for Rainbow Trout Tested Under Flow-Through Exposure Conditions

Nominal[a] Concentration (mg/ℓ)	0 hr.		48 hr.		96 hr.		144 hr.		192 hr.	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
0.67	0/10	0	0/10	0	6/10	60	10/10	100	10/10	100
0.36	0/10	0	0/10	0	1/10	10	7/10	70	9/10	90
0.24	0/10	0	0/10	0	1/10	10	1/10	10	1/10	10
0.11	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
Control	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0

Table 15

Cumulative Mortality Data for Daphnia magna (1st instar) in Hard Water During Static-Renewal Chronic Exposure

Nominal[a] Concentration (mg/ℓ)	0 days		7 days		14 days		21 days	
	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)	no. dead / no. exposed	mortality (%)
0.16	0/15	0	3/15	20	14/15	93	15/15	100
0.08	0/15	0	0/15	0	3/15	20	8/15	53
0.04	0/15	0	1/15	7	3/15	20	4/15	27
0.02	0/15	0	1/15	7	3/15	20	4/15	27
0.01	0/15	0	1/15	7	2/15	13	3/15	20
Ethanol Control	0/15	0	0/15	0	0/15	0	1/15	7
Control	0/15	0	1/15	7	2/15	13	3/15	20

[a]Each PNA concentration and ethanol control contained 0.2 mℓ ethanol/ℓ water.

Table 16

Test Conditions, LC50 and NOEC Values for Fish and Waterfleas Exposed to PNA.

Species	Age or Size	Test[a] Method	Water[b] Hardness (mg/l as $CaCO_3$)	Temperature (°C)	LC50 and NOEC Values (mg/l)[c] 2 days LC50	2 days NOEC	4 days LC50	4 days NOEC	6 days LC50	6 days NOEC	8 days LC50	8 days NOEC	14 days LC50	14 days NOEC	21 days LC50	21 days NOEC
Rainbow Trout *Salmo gairdneri*	0.6 g	SR	118	13 ± 2	0.52 (0.44-0.61)	0.36	0.44 (0.40-0.49)	0.22	-	-	-	-	-	-	-	-
	4.4 g	FT	118	13 ± 2	>0.60	0.17	0.74 (0.36-1.54)	0.11	0.37 (0.26-0.51)	0.12	0.30	0.11	-	-	-	-
Bluegill Sunfish *Lepomis macrochirus*	1.0 g	SR	118	20 ± 2	>1.00	0.60	0.82 (0.65-1.03)	0.36	0.63 (0.53-0.75)	0.36	0.52 (0.45-0.59)	0.22	-	-	-	-
	1.8 g	FT (Test 1)	118	20 ± 2	>0.62	0.62	>0.57	0.37	0.44 (0.35-0.56)	0.18	0.46	0.18	-	-	-	-
	1.8 g	FT (Test 2)	118	20 ± 2	>1.02	0.39	0.82	0.38	0.51	0.24	0.48	0.24	-	-	-	-
Waterflea *Daphnia magna*	Adult	S	44	20 ± 2	0.68 (0.53-0.88)	0.22	-	-	-	-	-	-	-	-	-	-
	1st instar	S	44	20 ± 2	0.30 (0.25-0.35)	0.13	-	-	-	-	-	-	-	-	-	-
	1st instar	SR	44	20 ± 2	0.67 (0.50-0.90)	0.22	-	-	-	-	-	-	-	-	-	-
	1st instar	SR	170	20 ± 2	0.68 (0.53-0.88)	0.16	-	-	-	-	-	-	-	-	-	-
	1st instar	SR	170	20 ± 2	>0.16	0.16	>0.16	0.16	>0.16	0.08	> 0.16	0.08	0.10 (0.08-0.13)	0.02	0.06 (0.04-0.09)	0.02

[a] SR = static-renewal, FT = flow-through, S = static

[b] Water of 3 levels of hardness was used: 118 mg/l (tap water), 44 mg/l (EPA soft water), 170 mg/l (EPA hard water)

[c] The 95% confidence interval of the LC50 is given in parentheses when calculable. The NOEC is the highest test concentration which had no lethal or sublethal effect.

As shown in Table 16, the 96-hr LC_{50} of PNA for trout and sunfish ranged from 0.44 to 0.82 mg/ℓ. The 96-hr LC_{50} for trout was somewhat higher in the flow-through test than in the static-renewal test, although the LC_{50} values were not significantly different. The static-renewal 96-hr LC_{50} for rainbow trout was significantly lower than the static-renewal 96-hr LC_{50} for sunfish, indicating that rainbow trout may be more sensitive than sunfish to PNA. There was, however, no appreciable difference between the flow-through 96-hr LC_{50} values for the two species. The LC_{50} values for both species decreased with continued exposure time. The flow-through LC_{50} for trout decreased from 0.74 mg/ℓ at 4 days to 0.30 mg/ℓ at 8 days. The flow-through and static-renewal LC_{50} values for sunfish decreased from 0.82 mg/ℓ at 4 days to 0.46-0.52 mg/ℓ at 8 days. The mean 8-day LC_{50} for sunfish was 0.49 mg/ℓ, which is not appreciably different from the 8-day LC_{50} (0.30 mg/ℓ) for trout.

The static tests with adult and first instar daphnids in soft water indicated that the younger animals had a significantly lower 48-hr LC_{50} (Table 18). The LC_{50} values were 0.68 mg/ℓ for adults and 0.30 mg/ℓ for young. Subsequent static-renewal tests, however, showed that the 48-hr static-renewal LC_{50} for first instar daphnids in soft water (0.67 mg/ℓ) was nearly identical to that of adult daphnids in the static test (0.68 mg/ℓ) and higher than that of first instar animals tested under static conditions. In addition, the static-renewal 48-hr LC_{50} values were identical in both soft and hard water. These results indicate that water hardness does not affect the acute toxicity of PNA to daphnids and that there is little, if any, difference in sensitivity between first instar and adult D. magna to this toxicant.

Comparison of the 48-hr LC_{50} values for all three species indicates that D. magna and trout were equally sensitive to the toxic effects of PNA

at this exposure period. Although the 48-hr LC_{50} values for sunfish were higher than the highest test concentrations, the results suggest that daphnids and trout were somewhat more sensitive than sunfish at this time. Comparison of NOEC values at the longer exposure times in the chronic D. magna test and the fish tests supports the conclusion that daphnids and trout were equally sensitive to PNA and more sensitive than sunfish. The 4 day NOEC values were 0.16 mg/ℓ for daphnids, 0.11-0.22 mg/ℓ for trout and 0.36-0.38 mg/ℓ for sunfish. The 8-day NOEC values were 0.08 mg/ℓ for daphnids, 0.11 mg/ℓ for trout and 0.18-0.24 mg/ℓ (mean = 0.21 mg/ℓ) for sunfish.

Continued static-renewal exposure during the D. magna chronic test resulted in a 21-day LC_{50} of 0.06 mg/ℓ, which is about 1/10 of the acute 48-hr LC_{50} (0.68 mg/ℓ) for this species. The highest non-lethal concentration and lowest lethal concentrations were 0.02 and 0.04 mg/ℓ, respectively, indicating that some chronic toxic effects occurred at a PNA concentration of as low as 0.04 mg/ℓ.

The results indicate that the acute LC_{50} values of PNA for D. magna, bluegill sunfish, and rainbow trout ranged between 0.30 and 0.82 mg/ℓ. The lowest test concentrations which produced some toxic effects during 8 days of exposure ranged from 0.16 mg/ℓ for D. magna to 0.24 mg/ℓ for trout to 0.22-0.35 mg/ℓ for sunfish.

These results indicate that PNA has a high toxicity to aquatic organisms. There are very few other reports of PNA toxicity in aquatic animals. Applegate et al. (12) reported that 24 hr of static exposure to 5.0 mg/ℓ of PNA had no effect on larval lampreys (Petromyzon marinus) and fingerling rainbow trout and bluegill sunfish. These results were in contrast to the results of the present research because toxic effects were observed in our tests among rainbow trout and bluegill sunfish exposed for 24 hr to as much as 1.0 mg/ℓ. The

fish used by Applegate et al. (11) however, were much larger (∿ 4 inches vs. 1-2 inches) than those used in the present study. It is well established that larger fish are generally less sensitive than smaller fish of the same species. The difference in size may account for the different results.

Other studies of PNA toxicity to aquatic organisms include those of Epstein et al. (12) with a protozoa, of Sherfig and Dixon (13) with an alga, and of Greenhouse (14) with a frog species. Epstein et al. (12) studied the effects of the commercial PNA mixture (Neozone A) on the reproduction of a freshwater protozoan (Tetrahymena pyriformis). The 48-hr ID_{50} (median inhibitory dose, based on cell numbers) was 2 mg/ℓ. Scherfig and Dixon (13) reported that exposure to 100 mg/ℓ PNA in a high nutrient medium decreased the growth of algae (Selenastrum capricornutum) by 50%. Greenhouse (14) reported that the 48-hr LC_{50} for larvae of the frog, Xenopus laevis, was 2.3 mg/ℓ. In addition, Greenhouse found that PNA was teratogenic to X. laevis embryos. The EC_{50} (median effective concentration), based on the number of deformed embryos, was 4.57 mg/ℓ.

Although these toxicity values are higher than those obtained in the present study, the available data indicate that acute toxicity to aquatic organisms can occur at PNA concentrations as low as about 0.1 mg/ℓ and that chronic toxicity may occur at much lower concentrations (∿0.04 mg/ℓ). Mammals seem to be much less sensitive to the toxic effects of PNA. The oral LD_{50} (median lethal dose) values for rats and mice were 1625 mg/kg and 1231 mg/kg, respectively (15)

Although PNA is highly toxic to aquatic organisms, an aquatic hazard assessment must consider whether PNA reaches toxic concentrations in polluted environments. Very little information is available concerning PNA concentrations in natural aquatic habitats. The only reports of PNA in a polluted aquatic

habitat are those of Jungclaus et al (16) and Lopez-Avila and Hites (17) who detected PNA in the effluent of a specialty chemical manufacturing plant and in the water and sediments of the river receiving these effluents. Lopez-Avila and Hites (17) reported PNA concentrations of 0.04-1.0 mg/ℓ in plant waste water, up to 0.01 mg/ℓ in river water and 1-5 mg/ℓ in river sediments.

Although no other instances of PNA pollution were found in the literature, the monitoring data from this study indicate that PNA can reach concentrations in effluents, sediments and river water which may cause toxic effects to aquatic organisms. As described in the next section of this report, the high bioconcentration potential of PNA suggests that consumption of aquatic organisms from such polluted areas by wildlife and humans may be hazardous.

UPTAKE, ELIMINATION & METABOLISM OF PNA BY FISH

Uptake of ^{14}C-PNA

In order to have some preliminary information on the bioconcentration of PNA in fish, uptake of the chemical was examined under static exposure conditions for a relatively short period. Subsequently, the uptake and elimination of PNA by the fish were determined over an extended period under flow-through exposure conditions.

Static Exposure Studies

The results of the preliminary static exposure experiments are given in Tables 17 and 18. The data in these experiments were based on dry tissue weights and, therefore, have been converted to wet weight by assuming an average 80% water content in fish tissue. These preliminary results indicated that PNA was accumulated rapidly in fish and that viscera accumulated much more PNA than did the edible portion. In addition, there was no appreciable difference in bioconcentration

factor between the two PNA exposure concentrations. The preliminary elimination experiment indicated that PNA was eliminated at two rates which were similar for each tissue (Table 19). About 50% of the radioactivity was eliminated at an initial fast rate in about 6-10 hours after transfer to clean flowing water. About 50% of the radioactivity remaining after 24 hours was eliminated at a slower rate in about 3 days.

Table 17

Uptake of ^{14}C-PNA by Bluegill Sunfish under Static Exposure Conditions[a]

	ppm ^{14}C-PNA equivalent[b]							
	Viscera		Head		Edible Flesh		Water	
Hours	0.2 mg/ℓ	.02 mg/ℓ	0.2 mg/ℓ	.02 mg/ℓ	0.2 mg/ℓ	.02 mg/ℓ	0.2 mg/ℓ	.02 mg/ℓ
7	70	9	38	3	18	2.0	0.163	0.016
24	160	22	52	4	35	3	0.115	0.011
48	265	40	67	4	36	2	0.104	0.010

[a] Fisher were exposed in water containing initial ^{14}C-PNA concentrations of 0.2 mg/ℓ (spec. act. = 2525 dpm/µg) or 0.02 mg/ℓ (spec. act = 27,250 dpm/µg PNA).

[b] Values are the average of 2 to 4 fish samples or 6 water samples.

Table 18

Bioconcentration of ^{14}C-PNA in Bluegill Sunfish Under Static Exposure Conditions

Tissue Fraction	ppm ^{14}C-PNA equivalent at 48 hr[a]		Bioconcentration[b] Factor
	in fish tissue	in water	
0.2 ppm PNA (initial)			
Viscera	265	0.104	2548
Head	67		644
Edible Flesh	36		346
0.02 ppm PNA (initial)			
Viscera	40	0.010	4000
Head	4		400
Edible Flesh	2		200

[a] Values are the average of 2 to 4 fish samples or 6 water samples.

[b] Bioconcentration factor = ppm ^{14}C-PNA equivalent in fish (wet wt.)/ppm ^{14}C-PNA equivalent in water.

Table 19

Elimination of ^{14}C-PNA by Bluegill Sunfish after Static Exposure[a]

Depuration Time (hours)	Percent ^{14}C-PNA retained in fish tissue		
	Viscera	Head	Edible Flesh
6	54	42	56
24	42	29	36
168	18	16	26

[a] The fish were exposed for 5 days to an initial concentration of 0.2 mg/ℓ ^{14}C-PNA and then transferred to clean flowing water. Mean initial ^{14}C-residues at the time of transfer to PNA-free water were 2141, 233 and 124 ppm ^{14}C-PNA equivalent in viscera, head and edible flesh, respectively.

Flow-through Exposure Studies

The uptake of ^{14}C-PNA and elimination of ^{14}C-PNA by the fish exposed to a sublethal concentration of the chemical are shown in Figure 11. PNA was readily taken up by the fish and an equilibrium between concentration of ^{14}C-material in the fish and exposure water was apparently reached between 8 and 10 days. At this time the bioconcentration factors (based on total ^{14}C-residues) in the whole fish, edible flesh and viscera were 1111, 627, and 3820, respectively (Table 20). HPLC analysis of the extracts of 10 fish sampled at the end of the uptake period indicated that 54 $\pm$ 17% (mean $\pm$ S.D.) of the extractable radioactivity was in the form of unaltered PNA at equilibrium. The bioconcentration factors (based on PNA concentration) in the whole fish, edible flesh, and viscera were 600, 227, and 1424, respectively. The uptake rate constants (whole fish), derived according to the model described by Branson et al. (18) was estimated to be 14.7 hr^{-1}.

The distribution of ^{14}C within the fish tissues after 10 days of exposure to ^{14}C-PNA is shown in Table 20. As noticed in the static exposure experiment, the concentration of ^{14}C-material in the viscera was substantially higher than in the edible flesh.

An empirical relationship has been established between the octanol/water partition coefficient (P) or organic chemicals and the bioconcentration factor (BCF) in fish muscle (19) and whole fish (20). The equations, respectively, are:

$$\log BCF = 0.85 \log P - 0.70 \quad (19)$$

$$\log BCF = 0.542 \log P + 0.124 \quad (20)$$

The octanol/water partition coefficient of PNA, as determined in this laboratory, is 16918 (log P = 4.228). Thus, the equations predict a BCF for fish muscle of 260 and a BCF for whole fish of 783. The experimental BCF values

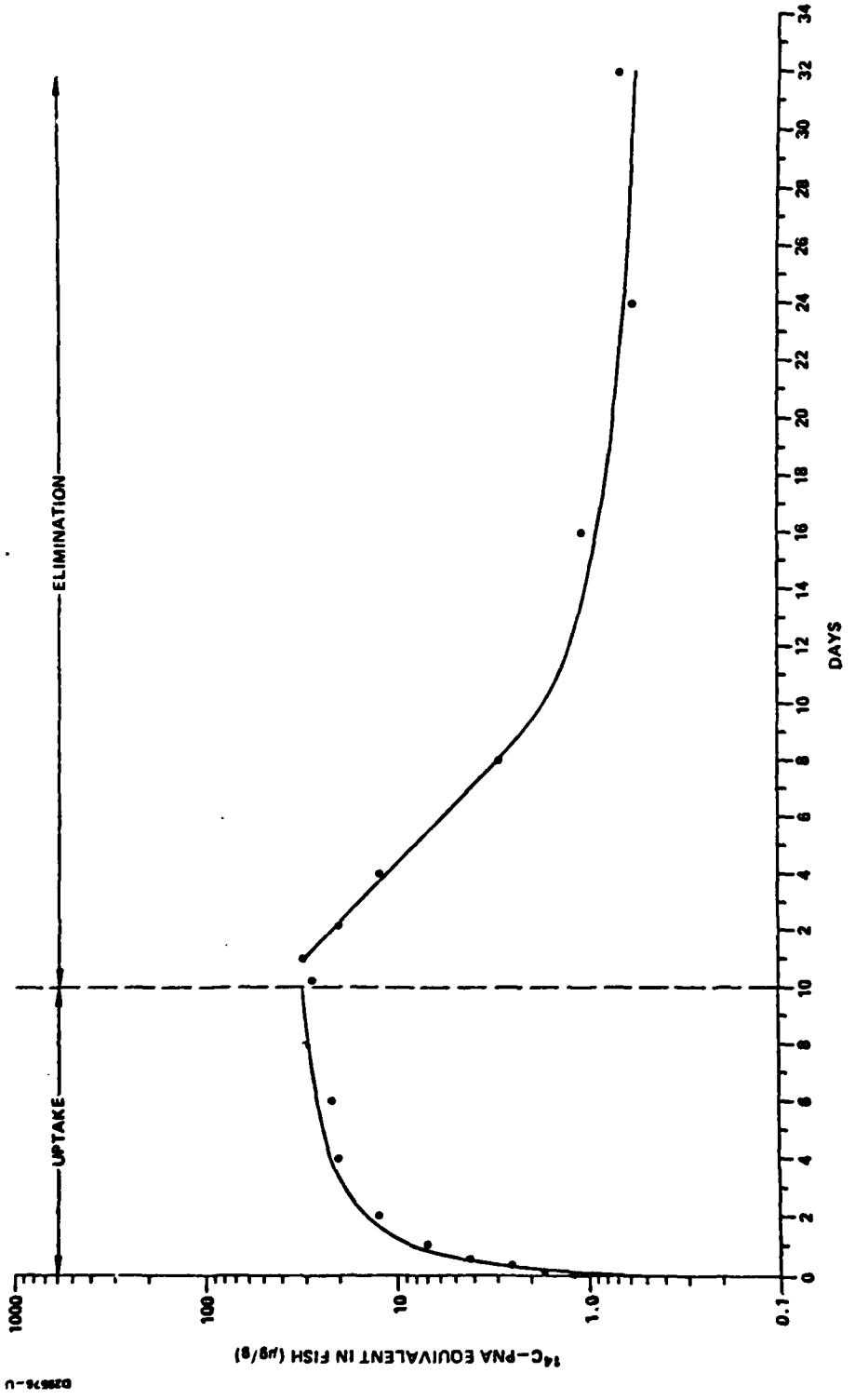


Figure 11. Uptake and elimination of ^{14}C-PNA in Bluegill Sunfish

Table 20

Bioconcentration of PNA in Bluegill Sunfish Under Flow-Through Exposure Conditions[a]

Tissue Fraction	ppm ^{14}C-PNA equivalent at equilibrium	ppm PNA at[b] equilibrium	Bioconcentration Factor	
			Based on total ^{14}C (PNA equivalent)	Based on PNA
Whole Fish	33.3 (28.0 - 38.6)	18.0 (15.1 - 20.8)	1111 (432 - 1285)	600 (233 - 694)
Edible Flesh	18.8 (15.1 - 21.6)	10.2 (8.2 - 11.7)	627 (503 - 720)	339 (272 - 389)
Viscera	114.6 (91.6 - 137.6)	61.9 (49.5 - 74.3)	3820 (2053 - 4587)	2063 (1109 - 2477)

[a]Numbers represent the mean ± 1 S.D. of fish samples at equilibrium.

[b]Calculated as 54% of total ^{14}C-resudue (see text).

were 227 for edible flesh and 600 for whole fish. The predicted and experimental values are in good agreement with each other.

Elimination of ^{14}C-PNA-Derived Radioactivity

Figure 11 shows the elimination of ^{14}C-PNA-derived radioactivity from the fish after their transfer to clean flowing-water. Elimination of ^{14}C-residue from the fish followed first-order kinetics until about 8 days after transfer to clean water. By this time more than 90% of the accumulated radioactivity had been eliminated. The elimination rate constant for this phase was estimated to be 0.014 hr^{-1}. The half-time for elimination phase was estimated to be 50 hrs.

After about 8 days, ^{14}C-residue in the fish decreased at a slower rate and ^{14}C-residue could still be detected 32 days after transfer of the fish to clean water. These findings show that ^{14}C-PNA (and metabolites) are rapidly eliminated from the fish. However, a small portion of the chemical residue seems to be tightly bound to cellular constituents and is resistant to elimination.

Metabolism of PNA by Bluegill Sunfish

HPLC analysis of the methanol extracts of the fish exposed to ^{14}C-PNA for 8 days showed at least 3 metabolites besides the parent compound (Figure 12). Approximately 60% of the radioactivity in the extract was present as unmetabolized PNA. Analysis of the methanol extract by combined GLC-MS showed the presence of a compound having a parent ion mass of 252 (Figure 13) which suggests that the metabolite is a hydroxy derivative of PNA. While the positions of the hydroxy groups in the molecule cannot be assigned by mass spectrum alone, it is likely that the metabolite is 4-hydroxyphenyl-1(4-hydroxynaphthyl)amine based on the metabolism in mammals of a structurally related aromatic amine, diphenylamine (21). However, isomeric structures with both hydroxy groups in the same ring or a hydroxy group

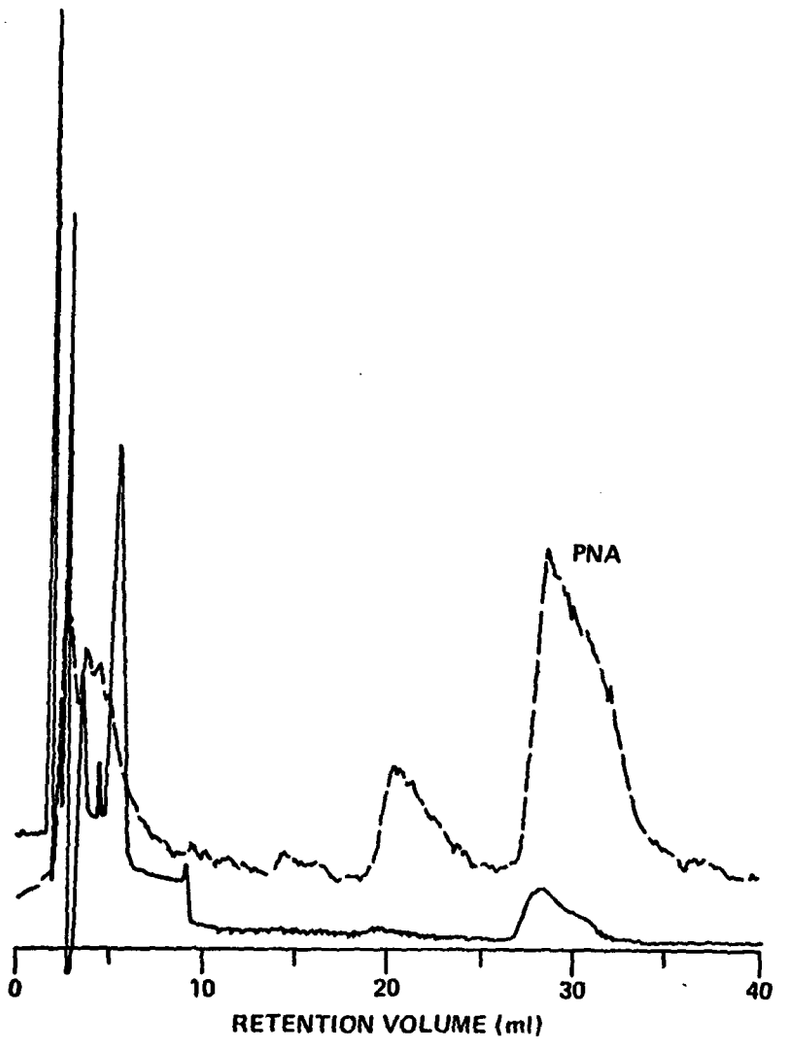


Figure 12. HPLC Profile of the Extract from Bluegill Sunfish Exposed to ^{14}C-PNA. Radioactivity Flow Detector (-); UV Detector (---).

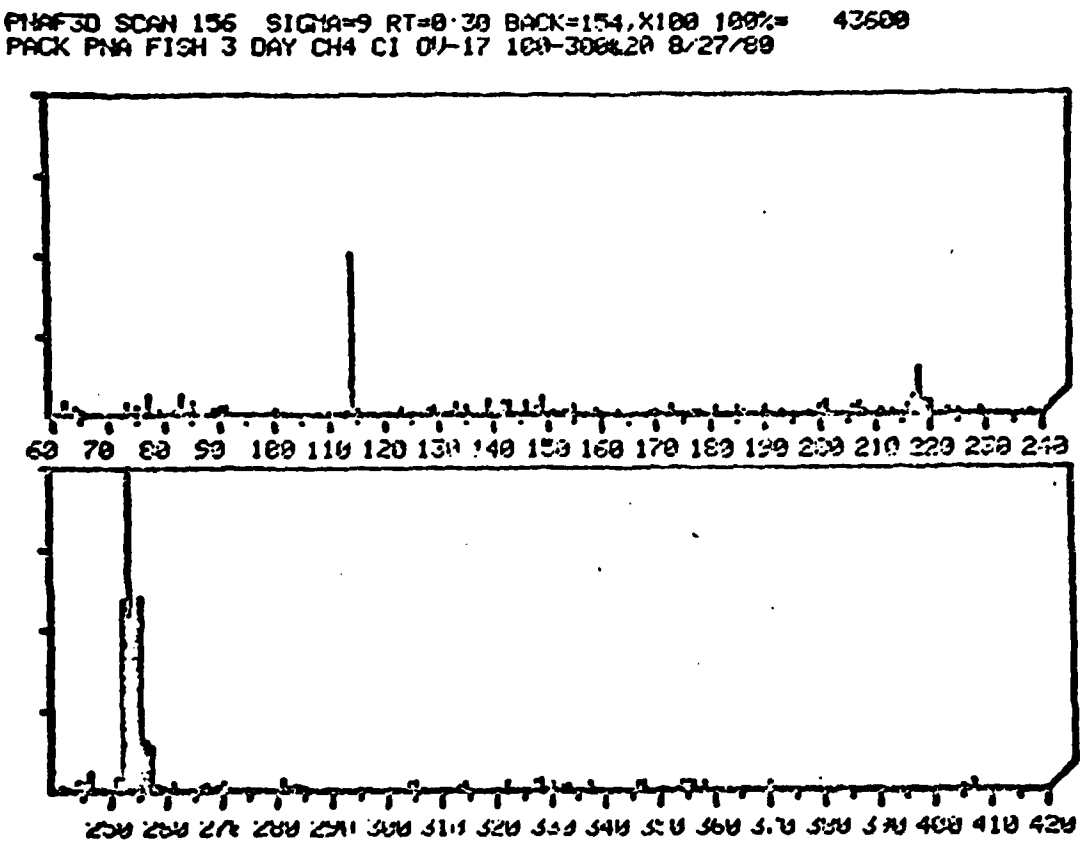


Figure 13. Mass spectrum of dihydroxy PNA formed during the metabolism of PNA by fish.

in each ring of the naphthalene moiety cannot be ruled out. The product probably results of the oxidative metabolism of PNA by fish microsomal enzymes. Enzymes catalyzing hydroxylation and other oxidative modifications of xenobiotics are known to occur in fish (22).

UPTAKE AND ELIMINATION OF PNA BY DAPHNIA

The results on the uptake and elimination of ^{14}C-PNA by D. magna exposed to a sublethal concentration of the chemical under static conditions are shown in Figure 14. PNA was taken up more rapidly by the daphnids than by fish and reached equilibrium by 12 hours, as shown by equal residue levels from 12 hours to the end of the exposure period at 72 hours. The average bioconcentration factor (based on total ^{14}C concentrations) during the 12-72 hours equilibrium period was 637. Elimination of ^{14}C-residue from the daphnids appeared to follow first-order kinetics until the end of the elimination phase at 53 hours, at which time about 50% of the accumulated radioactivity had been eliminated. The elimination rate constant was estimated to be 0.016 hr^{-1}, which is nearly identical to that obtained with fish.

The uptake rate constant, derived according to the reversible model described by Branson et al. (18) and calculated by multiplying the measured BCF (637) by the elimination rate constant (.016 hr^{-1}), was estimated to be 10.2 hr^{-1}. Use of these rate constants to calculate the time to half equilibrium ($BCF_{t\ 1/2}/BCF_{eq.} = 0.5 = 1 - e^{-k_2 t}$) yields a time to half equilibrium of 43 hours. As seen in Figure 13, the actual time to half equilibrium was only about 2 hours. The results indicate that the reversible model inadequately describes the uptake and release of PNA by D. magna, suggesting that some degree of irreversible binding may have occurred. Although use of the elimination rate constant of .016 hr^{-1} to extrapolate beyond the measured portion of the elimination phase suggests that daphnids

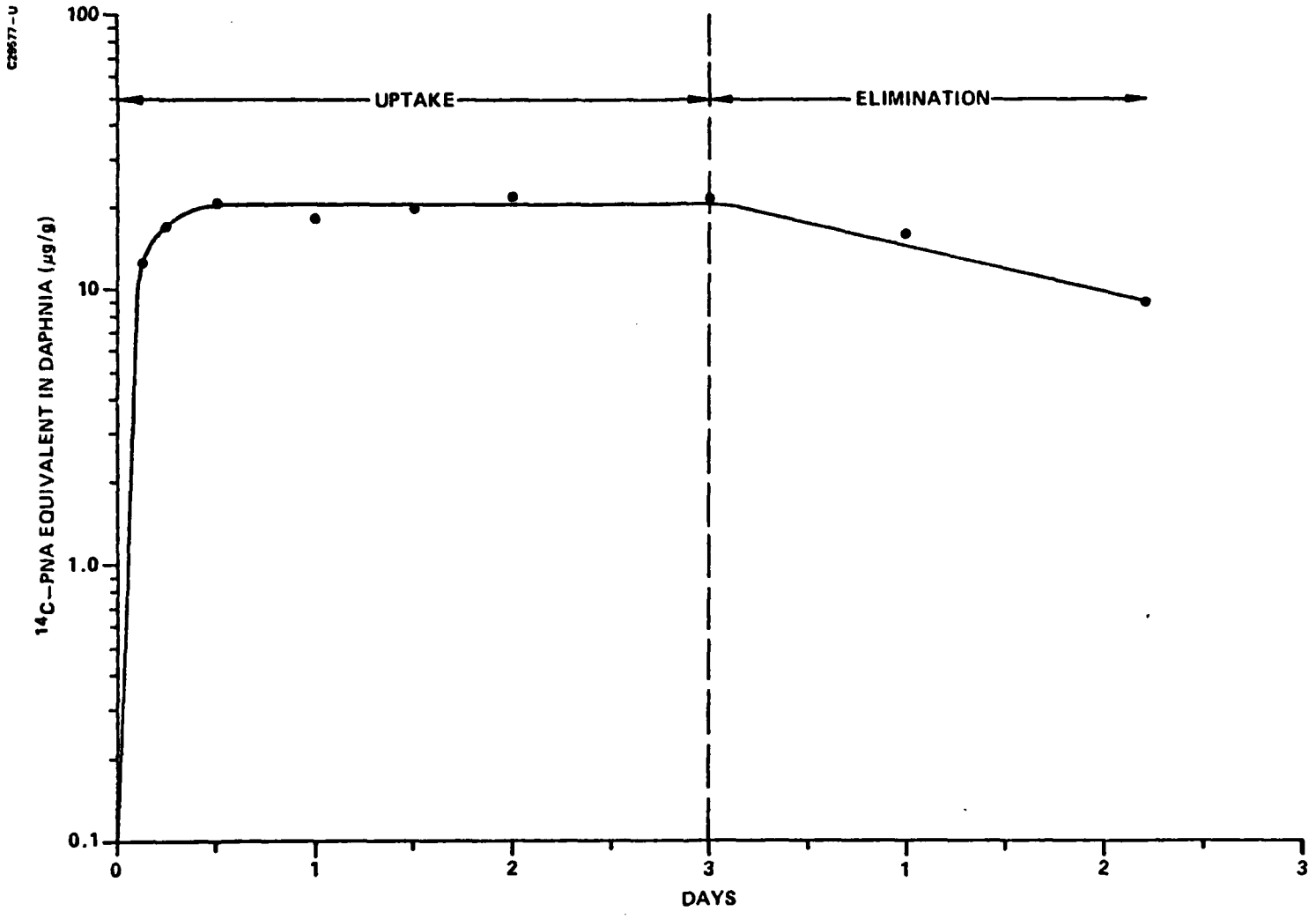


Figure 14. Uptake and elimination of ^{14}C-PNA in Daphnia magna

would eliminate 90% of the PNA residue by about 6 days, the possibility of irreversible binding indicates that PNA may be retained for a longer period of time in this species.

Our findings on the uptake of PNA by fish and invertebrates show that the chemical is likely to bioconcentrate to a significant extent in aquatic organisms. The BCF values reported here are of the same order of magnitude as the BCF of fathead minnows (Pimephales promelas) obtained with the PNA isomer, N-phenyl-2 naphthylamine, by Veith et al. (19). These authors reported an equilibrium (whole fish) BCF of 147 in fathead minnows exposed for 32 days under flow-through conditions.

Although PNA is bioconcentrated by aquatic organisms, an aquatic hazard assessment must consider whether fish and invertebrates in polluted environments attain PNA residue concentrations which may be hazardous for human consumption or to fish-eating wildlife. The only reports of PNA in a polluted aquatic habitat are those of Jungclaus et al. (16) and Lopez-Avila and Hites (17) who detected PNA in the effluent of a specialty chemical manufacturing plant and in the water and sediments of the river receiving these effluents. Lopez-Avila and Hites (17) reported PNA concentrations of 0.04-1.0 mg/ℓ in plant waste water, up to 0.01 mg/ℓ in river water and 1-5 mg/kg in river sediments. Fish exposed to 0.01 mg/ℓ of PNA in water would be expected to contain about 3.4 mg/kg PNA in edible flesh and 6.0 mg/kg in the whole body, based on the experimental BCF values of 339 and 600, respectively. These residues concentrations are three orders of magnitude below the median lethal oral doses reported for rats (LD_{50} = 1625 mg/kg) and mice (LD_{50} = 1231 mg/kg) by Lewis and Tatkin (15). However, benthic aquatic organisms, such as bivalve molluscs, would be exposed to much

higher concentrations of PNA in the sediments. Assuming that the BCF of bivalve molluscs would be about 600, it is possible that shellfish living in sediments containing 5 mg/kg PNA might accumulate up to 3000 mg/kg of this chemical. As a result, it is possible that the ability of PNA to be bioconcentrated by aquatic organisms may present a hazard to human and wildlife health through consumption of contaminated fish and shellfish.

DISCUSSION

Based on the results of our studies, it is obvious that photodegradation, microbial degradation, and bioaccumulation and biotransformation are among the processes which are likely to control the environmental fate and transport of PNA. These studies have only identified the likely pathways of PNA dissipation The contribution of each of these processes will vary with the ecosystems, however, since the rates of these processes are controlled by the environmenal conditions which are characteristic of an individual aquatic ecosystem.

PNA is rapidly degraded by sunlight and consequently is not expected to be present in surface waters in significant concentrations. While the photolytic half-life of PNA in clear distilled water may be as short as 6 minutes, this rate may not necessarily be representative of an entire natural aquatic system. In most cases, transparency, depth, degree of mixing and season would be critical factors in determining the rate at which PNA is degraded by sunlight.

Compared to photolysis, microbial degradation appears to be a less important process in governing the fate of PNA. Under our experimental conditions, half-life for biodegradation of PNA in lake water was approximately ten days. The rate of biodegradation was enhanced in the presence of an exogenous carbon and energy source which further suggests that the contribution of biodegradation will vary with the

dissolved organic content of aquatic ecosystem. Our results have demonstrated that biodegradation of PNA leads to mineralization of the molecule.

Although photolysis appears to be more effective in degrading PNA, the overall role of the two processes in removing the chemical from a natural aquatic ecosystem may be different from that noticed in the laboratory studies. Photodegradation of PNA would be a dominant process only in the uppermost photic zone, whereas microbial degradation would be expected to occur through the entire water column.

PNA is bioaccumulated by fish to a significant degree directly from contaminated water and may present a hazard to human health through consumption of contaminated fish. The hazard to humans will, however, depend upon whether the fish in polluted environments accumulate unsafe PNA concentrations.

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PART II. Effect of Sub-lethal Concentrations of PNA on the Feeding Behavior of Rainbow Trout

INTRODUCTION

Perturbations in behavior of aquatic organisms exposed to sublethal concentrations of xenobiotics have been documented (1,2,3,4). Relatively little is known, however, of detailed changes in feeding behavior resulting from exposure to toxic compounds. Optimal foraging theory (5,6), suggests that deviations in feeding behavior are likely to be reflected in reduced food intake, and/or increased energy expenditure. Presumably, subtle changes in feeding behavior may also be reflected in reduced ability of some species to compete for food resources. Because diet plays an important role in determining growth rate and ultimate size in fishes (7), changes in feeding pattern may influence rates of survival and reproduction.

Feeding patterns may be described in terms of number of prey eaten, prey size selection, distance of reaction to prey, rates of learning (or forgetting), etc. Ecologists have devoted much effort to the relationship of number of prey eaten to the number available. This measure is known as the "functional response", and may be expected to be linear ("Type I"), asymptotic ("Type II") or sigmoid ("Type III") (8,9). The shapes of the curves are significant because they indicate the predators' capacity to respond to changes in prey density. Exactly how behavioral events create the observed functional response requires a detailed analysis, including measurement of reaction distance, handling time, and satiation effects.

In a previous study by Ringler (10) an experimental procedure was developed to analyze behavior of drift-feeding brown trout (Salmo trutta). Ringler and Brodowski (11) reported a Type II functional response of brown trout to drifting invertebrate prey (Brine shrimp). This report presents

the results of a preliminary study on the effect of short-term sublethal PNA exposure on the functional response and reaction distance of adult rainbow trout (Salmo gairdneri)[1]. The major objectives were to determine whether PNA has the potential for causing subtle behavioral effects at concentrations that are not acutely lethal and to develop and test a protocol for future research. Because of the time constraint (6 weeks) we did not attempt to study a large number of individuals, or a great variety of PNA concentrations, although both would be essential in an exhaustive study.

MATERIALS AND METHODS

Adult rainbow trout ($\bar{x}$ total length: 150 mm, Range: 135 - 160 mm) were acquired as fingerlings from a private hatchery in Cazenovia, N.Y. They were held in cylindrical tanks at 13-14°C for approximately one year at the Syracuse Research Corp., Syracuse, N.Y. Individuals were exposed to 0, .04, .45 or .90 mg/ℓ PNA under static conditions at 13°C for 24 hours in 20ℓ glass containers. The PNA concentrations, respectively, were .1, 1, and 2 times the 96 hr. LC_{50} of PNA to rainbow trout fingerlings (see Section III). No mortalities or obvious toxic symptoms occurred during the 24 hr. exposure of adult rainbow trout to these concentrations. They were transported in a 60ℓ ice chest to the Fish Behavior Laboratory at the S.U.N.Y. College of Environmental Science and Forestry. Here they were conditioned to feeding on brine shrimp in a 605ℓ, eight-chambered trough. After one day, the three most active feeders were placed in separate sections of a 1370ℓ experimental stream (Fig. 1). Each

[1]This research was conducted in cooperation with Dr. N. Ringler (School of Biology, Chemistry, and Ecology, State University of New York, College of Environmental Science and Forestry, Syracuse, N.Y.)

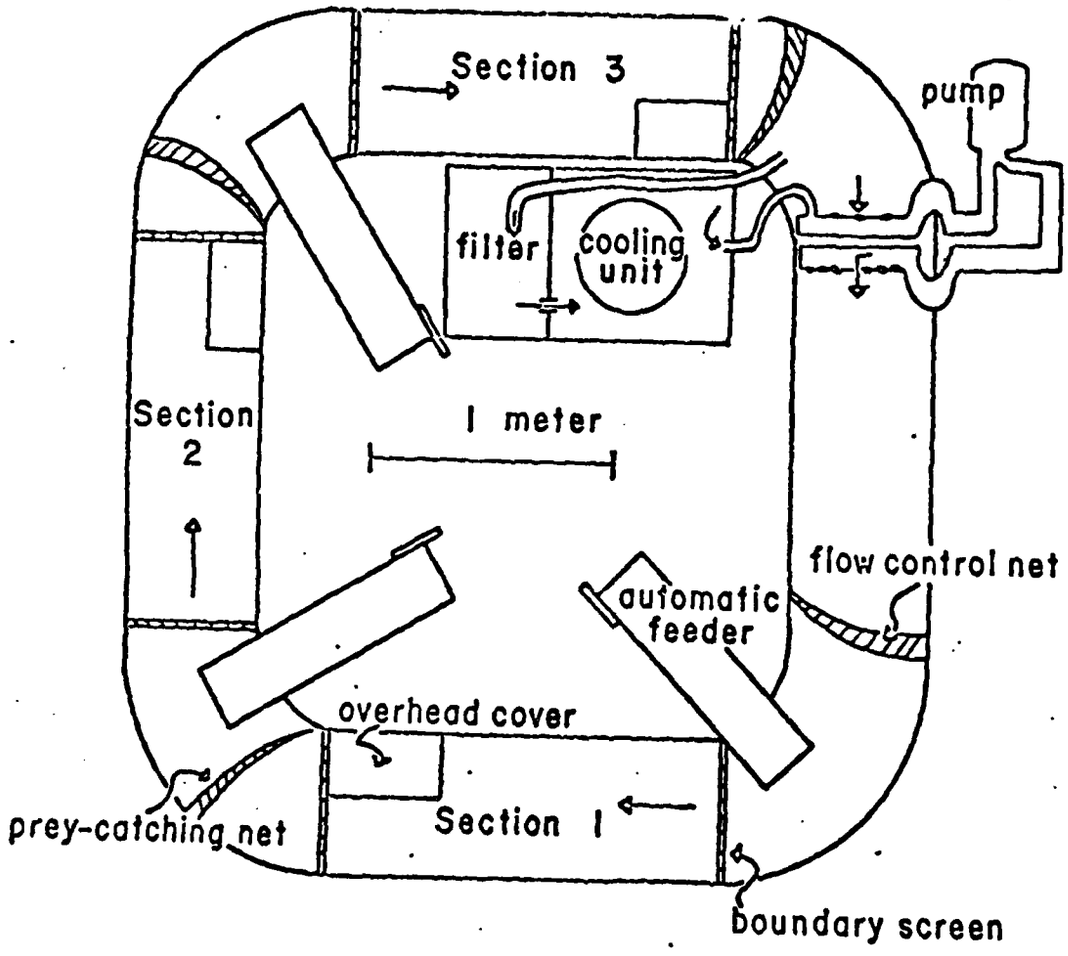

Figure 1. Schematic diagram of experimental stream

section was equipped with a window for observation and an automatic feeder. Water was circulated by means of an electric pump, providing a mean current velocity of 13 cm/s. Light intensity was maintained at 33 lux with a photoperiod of 15 hr. light : 9 hr. dark. Stream temperature was maintained at 16° ± 1°C. After a day in the stream, the fish were fed for 15 minute periods, twice a day for three days. A different concentration of food, assigned at random, was used for each feeding (Table 20). Randomization permitted an analysis of functional response to prey density, apart from effects attributable to learning to feed in the experimental stream. The total number of attacks, and their location with respect to a grid (marked on the face of the tank) was recorded with a 20-channel Esterline-Angus events recorder.

At the conclusion of the functional response experiment, the two most active feeders were chosen for measurement of reaction distance. Each fish was placed in a 150 x 43 cm tank filled to a depth of 30 cm. The fish were first exposed to the prey (mealworms; mean length = 19 mm) one day prior to the experiment. Each tank was equipped with a grid marked on the front wall; a 25 x 28 cm holding chamber isolated the fish from the experimental section. On the second day, a mealworm was placed on the floor of the tank, 95 to 115 cm from the door of the holding chamber. The fish was released and the distance from which it responded to the mealworm (i.e. changed direction or speed) was measured. The procedure was repeated for each fish, six times a day for three days. Our estimates of reaction distance were subject to some error, because in some trials, the fish attacked debris near the food or showed no obvious change in direction or speed prior to attacking the prey.

Table 1. Sequence (random) of food concentrations used to study the functional response of rainbow trout to brine shrimp. Numbers are estimates of total number of brine shrimp available (mean length 5 mm, range 2-11 mm) per 15 min., based on weight of frozen samples.

Treatment	Fish No.	DAY 1		DAY 2		DAY 3	
		AM	PM	AM	PM	AM	PM
Control	1	240	60	960	30	120	480
	2	30	960	480	120	240	60
	3	60	240	960	120	30	480
	4	480	240	120	60	30	960
	5	30	480	-	240	60	960
	6	240	960	30	60	480	120
0.04 ppm	7	240	480	960	60	30	120
	8	120	960	480	240	30	60
	9	480	30	120	960	240	60
0.45 ppm	10	120	30	480	960	240	60
	11	30	480	60	120	960	240
	12	240	480	960	120	60	30
	15	480	240	960	30	60	120
	16	30	960	60	240	480	120
	17	30	960	60	240	480	120
0.90 ppm	13	960	120	60	30	480	240
	14	240	960	120	480	60	30
	15	480	240	960	30	60	120

Table 2. Mean peak and mean total number of attacks on brine shrimp by rainbow trout exposed for 24 hr. to PNA.

	Control	0.04 ppm	0.45 ppm	0.90 ppm	Prob.
Peak No. attacked ($\bar{x}$)	160.33	138.33	84.60	137.33	$0.05<p<0.10$
Total No. attacked in 15 minutes ($\bar{x}$)	366.83	316.67	194.40	333.00	$0.10<p<0.20$
N	6	3	5	3	

RESULTS AND DISCUSSION

Comparisons of the mean peak feeding rates and the mean total attacks (over-all food concentrations) indicate that these means are lower in dosed groups, particularly in the third group (.45 ppm). However, these differences were not statistically significant (Table 2).

The functional responses of control rainbow trout conformed approximately to Type II, i.e., a decelerating rise to an asymptote (e.g. Fig. 2), although it seemed clear that higher prey densities could have been employed. We fitted the Holling (12) "disc" equation to these data as follows:

$$y = \frac{T_t \, a \, x}{1 + a \, b \, x}$$

Where y = no. prey attacked

x = prey density

T_t = experimental period (15 min)

a and b are fitted constants.

The functional response curves of control and exposed fish are shown in Figure 3. Analysis of the regression lines from which the curves were derived[1] showed significant differences ($p \leq .001$) among all slopes and, therefore, among the curves. However, no significance was found when comparing the groups individually using Dunnet's test (13). This was probably due to the small sample sizes creating too much variance within groups.

Differences in functional responses suggest that dosed fish have a lower rate of increase in feeding response to increased food concentrations, and

[1] Number of prey eaten/number prey available plotted against number eaten is a linear function that may be fitted by the method of least squares. We compared the slopes using analysis of covariance (13).

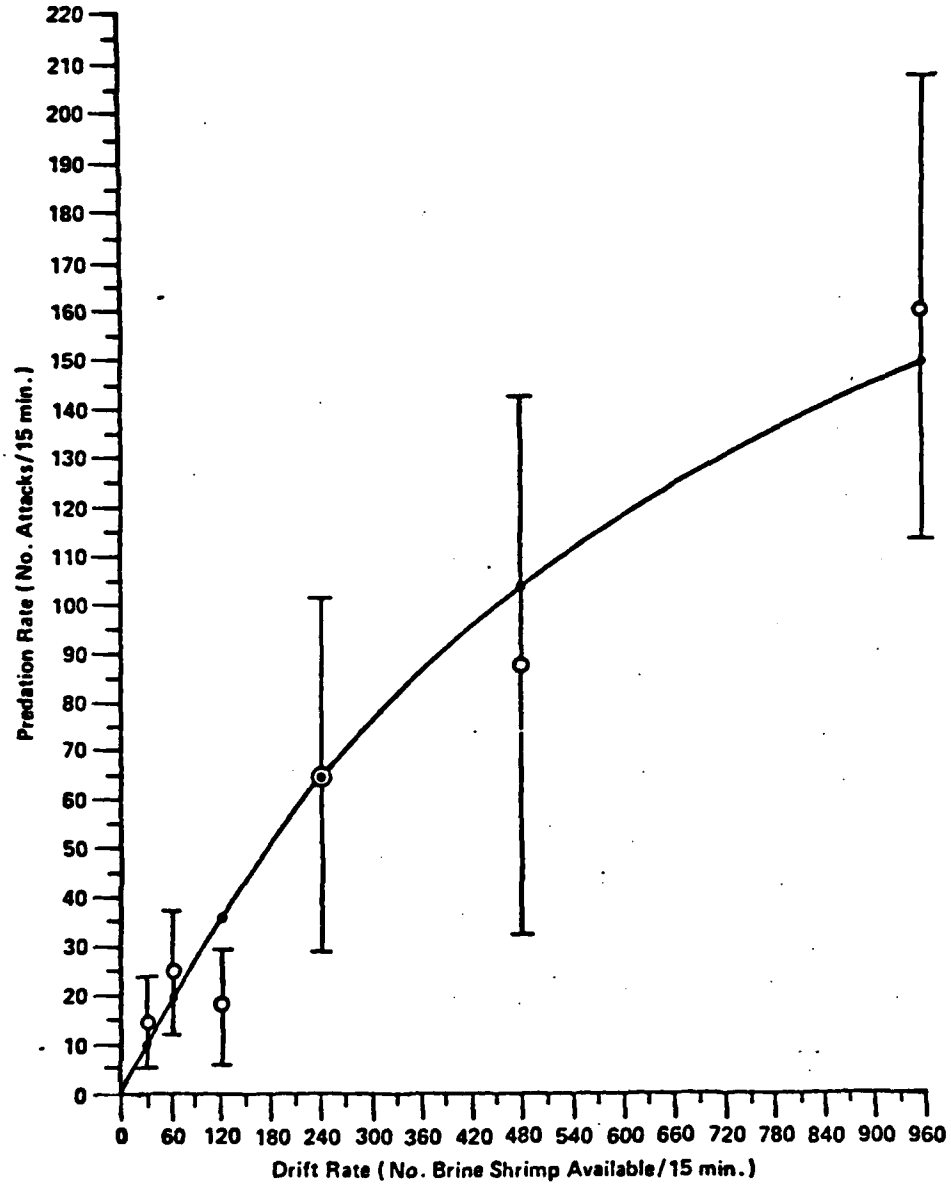

Fig. 2. Functional feeding response of control rainbow trout (N = 6). Open circles and bars represent mean ± 2 standard error. Points and curve were fitted from the data with the Holling equation (see Text).

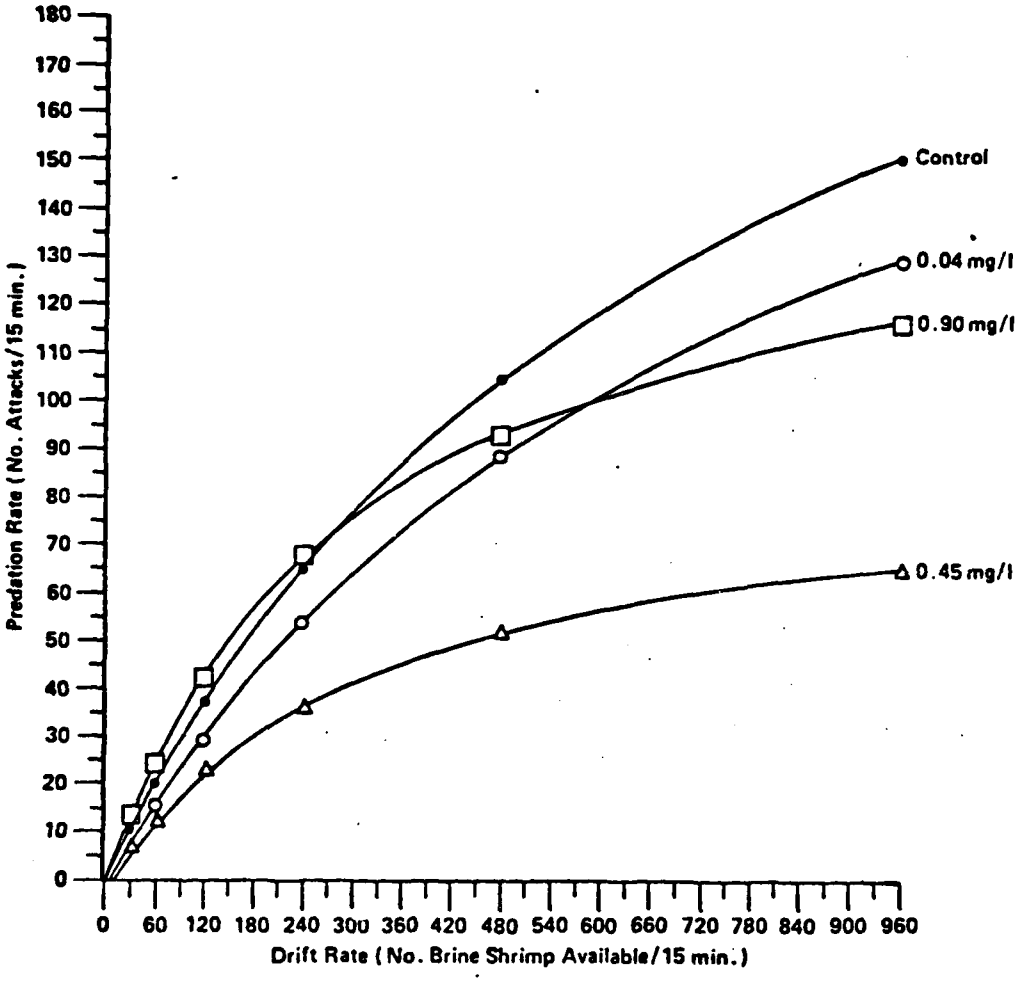

Fig. 3. Functional feeding response curves of control and PNA-exposed rainbow trout.

that they approach a lower maximum level. This may be caused by increased handling time and/or reduced rate of movement between attacks.

Measurements of reaction distance showed that the controls had a tendency to increase their reaction distance over time (Fig. 4). Mean distances for the second and third days were significantly different from those of the first ($p \leq .02$). The dosed fish, on the other hand, showed no significant difference between the means of each day. This indicates that the dosed fish may have a smaller capacity to learn than non-dosed fish. Sample size, however, should be larger and the fish should be tested over a longer period of time in order to more carefully measure learning capabilities. For example, Ware (14) found a change in response for up to 12 days, and unpublished data from Dr. Ringler's laboratory (15) suggests a change over at least a 5-day period.

Conclusions

We believe that a larger sample size, preferably n = 10 or more, would be required before generalizations could be made on the role of PNA on trout feeding. The available data suggest, however, that this toxicant may subtly change fish feeding behavior. If such effects occurred under natural conditions, we anticipate a decrease in growth-rate, and perhaps survivorship, of rainbow trout.

This study suggests that relatively simple measures of response to food may prove useful in evaluating the significance of toxic compounds to fish populations. Future studies of this type may provide opportunities to view toxicological investigations within an ecological framework.

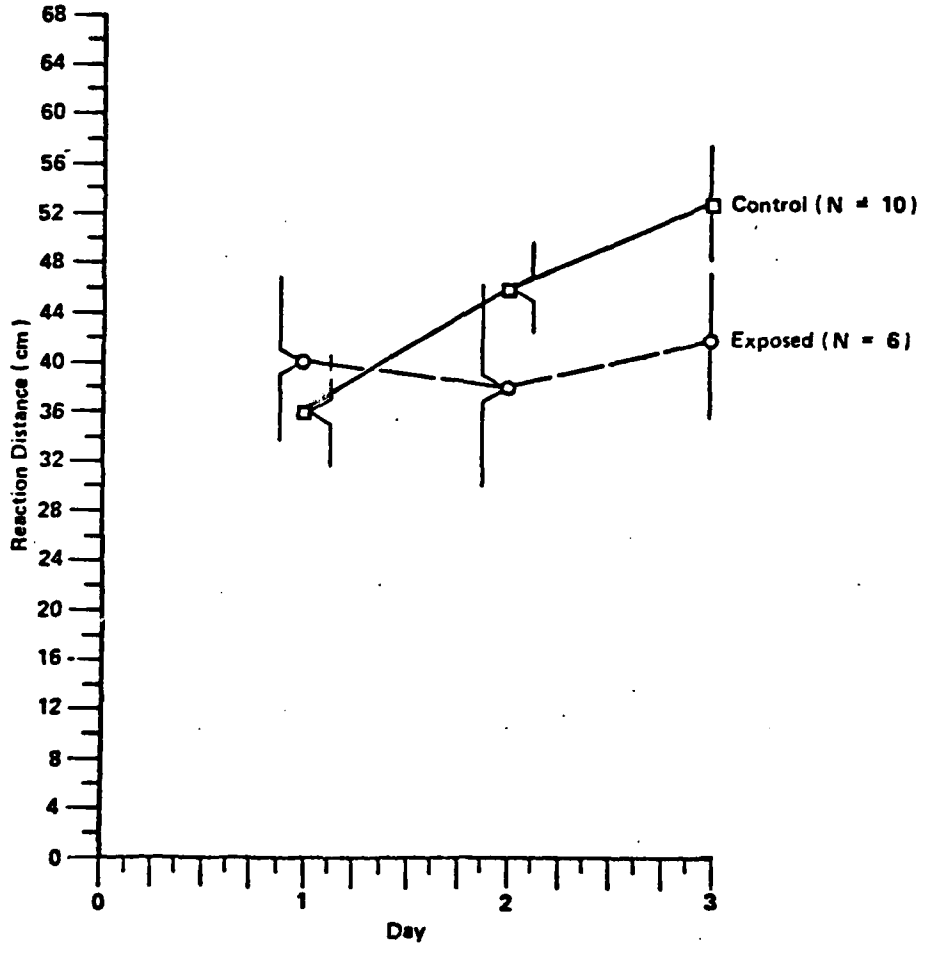

Fig. 4. Mean reaction distance of control and PNA-exposed (0.45 mg/ℓ) rainbow trout. Bars represent ± 2 standard error.

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PART III. Disposition and Metabolism of PNA in the Rat

INTRODUCTION

In our studies on the uptake of N-phenyl-1-naphthylamine (PNA) by bluegill sunfish, we have noticed that the chemical is bioaccumulated to a substantial amount by the fish. Therefore, in addition to occupational exposure to PNA, humans may potentially be exposed to the chemical in the environment. In order to assess the toxic potential of PNA to mammals, it is essential to have a knowledge of its behavior in the body (e.g. absorption, rate(s) and route(s) of excretion, overall persistence in the body (body burden), concentration in specific tissues, the extent of metabolism and the nature of metabolites). Since virtually nothing is known about the metabolic fate of PNA in mammals, we have investigated the disposition and metabolism of the chemical in rats.

SPECIFIC OBJECTIVES

1. To determine the absorption, excretion, and tissue distribution of PNA in rats.
2. To study the *in vivo* and *in vitro* metabolism of PNA in rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 225-250 g were purchased from Taconic Farms, Germantown, New York. The animals were kept under 12 hr light cycle for at least one week prior to use.

Radioassays

All radioassays were performed using a Packard Tri-Carb Model 3255 liquid scintillation spectrometer equipped with automatic external standardization. All data were corrected for background interference, quenching, and counting efficiency. Aliquots of water and organic extracts were counted in Instagel. Samples of feces and tissues were oxidized by combusting in a Packard Sample Oxidizer Model 306 using 10 mℓ of the trapping solution Carbo-Sorb and 12 mℓ of the scintillation cocktail Permafluor (Packard Instrument Company). ^{14}C-PNA and metabolites eluting from the HPLC column were detected and quantified by a radioactivity flow detector (Radiometric Instrument and Chemical Company, Inc., Addison, IL).

Excretion Studies

The animals were fasted for about 12 hr prior to administering PNA, but were allowed free access to water. The rats were given a dose (160 mg/kg) of ^{14}C-PNA dissolved in 0.25 mℓ of dimethylsufoxide (DMSO) by oral intubation; the animals were then housed in individual metabolism cages which permit separate collection of urine and feces. The urine and feces samples were collected at appropriate intervals over a period of 96 hr. At each collection period, duplicate aliquots of urine samples were assayed for total radioactivity and the remaining samples were frozen for later analysis. The feces samples were homogenized with added water to give a uniform paste, and triplicate aliquots were assayed for total radioactivity.

Kinetics of Plasma and Tissue Radioactivity

A group of fasted rats were given an oral dose of 160 mg/kg ^{14}C-PNA. The animals were killed in groups of three at 1/2, 1, 2, 4, 8, 12, 16, 24, 48 and 72 hr by exsanguination under ether and the blood was withdrawn with heparized syringes. The plasma was separated from the erythrocytes by centrifugation at 3000 x g for 10 minutes and an aliquot was counted for radioactivity. To determine the levels of PNA, an aliquot of the plasma was diluted with an equal volume of distilled water, adjusted to pH 10 with NaOH and extracted 3 times with diethyl ether. The pooled ether extracts were dried under a stream of nitrogen and the residue was dissolved in methanol. The methanol extract was analyzed by HPLC using a Varian high-pressure liquid chromatograph equipped with a UV detector, radioactivity flow detector and a μ-Bondapak C_{18} reversed phase column. The column was eluted with a linear solvent gradient from 20% acetonitrile:water to 80% acetonitrile:water at a flow rate of 2 mℓ/min.

The tissues were removed from the animals at the time periods described above and assayed for total radioactivity. The appearance and disappearance of the total radioactivity and parent compound in the plasma were analyzed by the stripping technique (1) utilizing a computer program to resolve the pharmacokinetic data into individual first-order components.

Metabolism of PNA

In Vivo Metabolism.

Pooled (0-24 hr) urine samples were adjusted to pH 12 with 2 N NaOH and extracted three times with diethyl ether. The remaining aqueous layer was adjusted to pH with 1 N HCl and extracted three times with diethyl ether. The combined ether extracts were counted for radioactivity and analyzed for ^{14}C-PNA and metabolites by HPLC as described above.

In Vitro Metabolism.

The in vitro metabolis of PNA was examined using rat liver microsomes. The microsomes were obtained from rats that had been given i.p. injections of Aroclor 1254 (500 mg/kg) four days prior to sacrifice. The microsomes were prepared according to the published procedures (2, 3). Freshly removed liver tissue was rinsed in cold (0-4°) 0.05 M phosphate buffer (pH 7.4) containing 1.15% KCl. The tissue was minced with scissors and homogenized with 3 volumes of cold buffer in a glass Potter-type homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged at 9000 x g for 20 minutes and the supernatant was centrifuged at 105,000 x g for 60 minutes. The pellet was resuspended in the 0.1 M phosphate buffer (pH 7.4) and centrifuged a second time at 105,000 x g for 60 minutes; the washed microsomes were then suspended in a volume of the buffer to produce the desired protein concentration.

The incubation mixture in a total volume of 100 mℓ contained 25 mg of microsomal protein, glucose-6-phosphate dehydrogenase (45 units) $NADP^+$ (52.6 mg), glucose-6-phosphate (98 mg), $MgCl_2$ (32 mg) and 3.8 mg of PNA dissolved in DMSO. The reaction mixture was incubated at 37°C for 3 hours. The incubation mixture was extracted twice with diethyl ether. The two extracts were combined, dried over anhydrous Na_2SO_4 and concentrated under vaccum and the residue dissolved in ∿ 1 mℓ of methanol for HPLC analysis. The metabolites were separated by HPLC on a Varian HPLC equipped with a μ-Bondapak C_{18} reversed phase column. The metabolites were eluted with a linear gradient of 60:40 acetonitrile:water to 100% acetonitrile. Fraction of the eluant corresponding to UV detected peaks were collected in clear vials. The solvent was removed under a stream of nitrogen at

< 40°C and the residue was dissolved in 0.01 mℓ of acetonitrile. The solution was then transferred to microvials, the solvent was removed under a stream of nitrogen (through teflon tubing) at <40°, and the metabolites were analyzed by direct inlet mass spectrometry.

RESULTS

Kinetics of ^{14}C-PNA Derived Radioactivity in Plasma

The concentration of ^{14}C-PNA-derived radioactivity and the parent compound in the plasma of rats following an oral dose of ^{14}C-PNA are shown in Figure 1 . The concentration of total radioactivity reached a peak of 116 µg PNA equivalents/ mℓ four hours after dosing, thereafter it declined in a biphasic manner. About 3% of the radioactivity in the plasma could be attributed to the parent compound 4 hours after dosing.

The appearance and disappearance of plasma radioactivity shown in Figure 1 was fitted to a pharmacokinetic two-compartment open model with first-order-absorption according to the following equation (1) where KA is

$$C = Ae^{-\alpha\tau} + Be^{\beta\tau} - De^{-KA\tau}$$

the first-order availability rate constant and α and β are the first-order elimination constants for the rapid and slower phases, respectively. Analysis of the plasma radioactivity according to this relationship gave half-life values of 1.59 hr for the appearance process and 1.68 and 33 hr, respectively, for the two elimination processes. The level of unchanged PNA in the plasma followed a kinetic pattern similar to that of the total ^{14}C but reached its peak level of 1.25 µg PNA/mℓ plasma at 2 hr after dosing, after which it declined in a biphasic manner. Analysis of the plasma levels of PNA gave half-life values of 0.66 hr for the appearance process and 1.24 and 11.1 hr, respectively, for the two disappearance processes.

Tissue Distribution

Twenty-four hours after the animals received a 160 mg/kg oral dose of ^{14}C-PNA, ^{14}C remaining in all of the major organs and tissues was determined. The data presented in Table 1 shows that appreciable radioactivity was detected in all

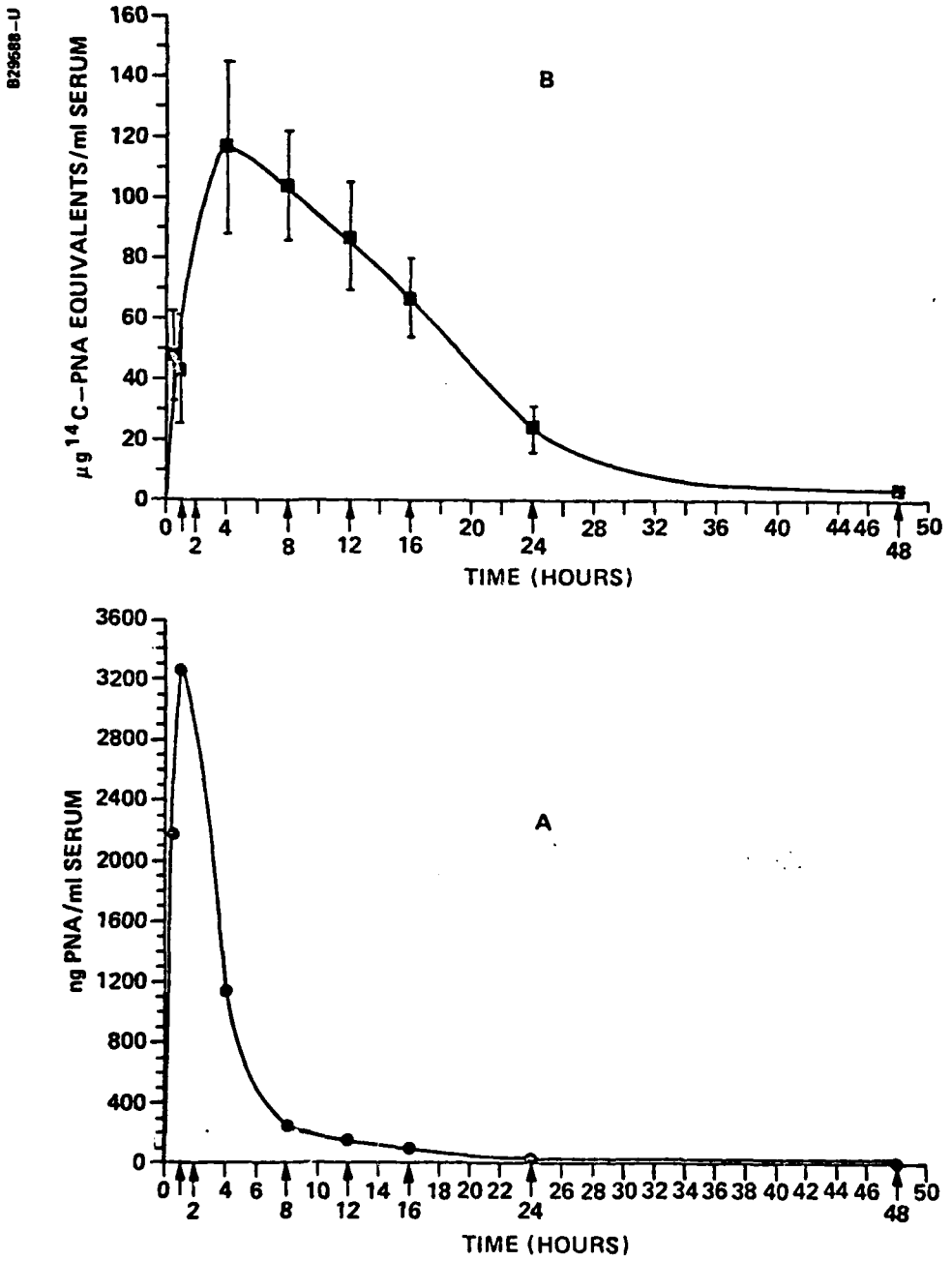

Figure 1. Concentration of PNA (A) and [^{14}C]PNA-Derived Radioactivity (B) in the Plasma of Rats Receiving An Oral Dose (160 mg/kg) of ^{14}C-PNA.

Table 1

Distribution of radioactivity in rat tissues 24 hours after oral administration of ^{14}C-PNA

Tissue	^{14}C-PNA Equivalent µg/gr Wet Tissue	Percent of Administered Dose
Heart	6.49	0.0130
Lung	10.2	0.0280
Pancreas	3.62	0.00432
Spleen	3.18	0.00377
Kidney	28.7	0.123
Testes	3.30	0.0200
Liver	33.8	0.415
Fat	66.0	2.35
Stomach	488	5.62
Small Intestine*	235	5.34
Caeca	362	5.67
Large Intestine*	435	3.10

*Includes contents.

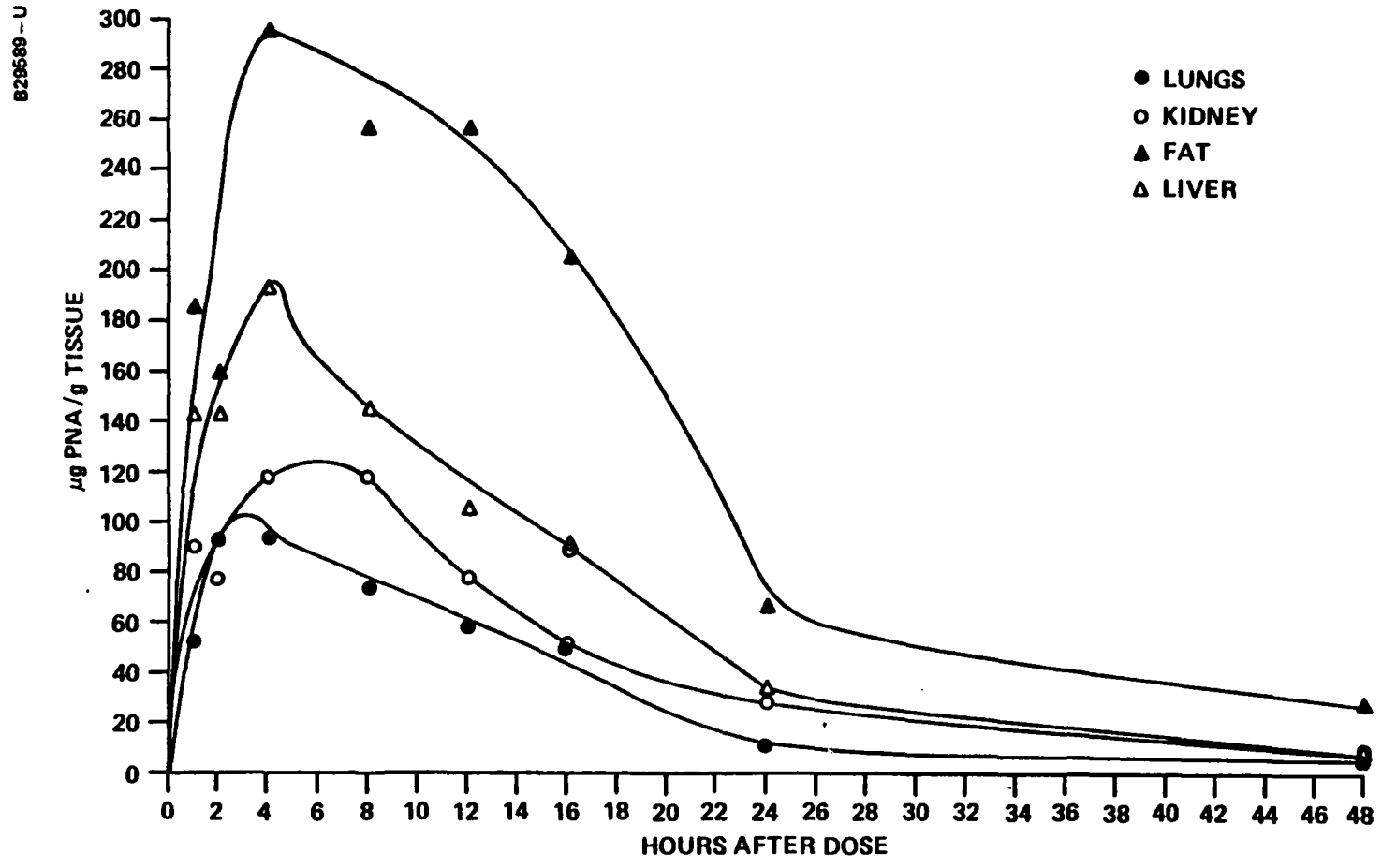

Figure 2. Concentration [^{14}C]PNA-derived radioactivity in fat, liver, kidney, and lung of rats administered an oral dose (160 mg/kg) of ^{14}C-PNA.

tissues at 24 hours indicating a rapid absorption and distribution of ^{14}C-PNA and its metabolites. The major sites of distribution of ^{14}C were fat, liver, kidney and lungs with large amounts in the gastro-intestinal tract plus contents.

Further studies were conducted to determine the distribution of radioactivity as a function of time in four major tissues depots (i.e., liver, fat, kidney, and lung) after an oral dose of ^{14}C-PNA. In all four tissues, radioactivity reached a maximum about four hours after dosing, reflecting a rapid uptake and distribution of the chemical (Figure 2). The levels of radioactivity then appeared to decrease in a biphasic manner. At all times, the highest concentration of ^{14}C were found in the liver followed by fat, kidney and lung. When radioactivity is expressed in terms of percent of the total dose that the animals received, only liver and fat are the important tissues which retained most of the unexcreted radioactivity.

Urinary and Fecal Excretion

Figure 3 shows the cumulative excretion of ^{14}C-PNA-derived radioactivity into the urine and feces of rats receiving an oral dose of ^{14}C-PNA. The data show that more than 90% of the administered dose had been excreted into the urine and feces within 48 hours of administration. The primary route of excretion of radioactivity was via the feces although appreciable amounts of ^{14}C was excreted via the urine. After 72 hours, 60% of the total dose was excreted in the feces and about 35% in the urine. The bulk of the excretion in the urine and feces occurred during the 24 hours after administering the chemical.

Metabolism of PNA

In Vivo Metabolism. In order to characterize the excreted radioactive material(s), the urine samples from the rats treated 48 hr earlier with ^{14}C-PNA were extracted with diethyl ether at pH 12 and 2. The percentage of urinary

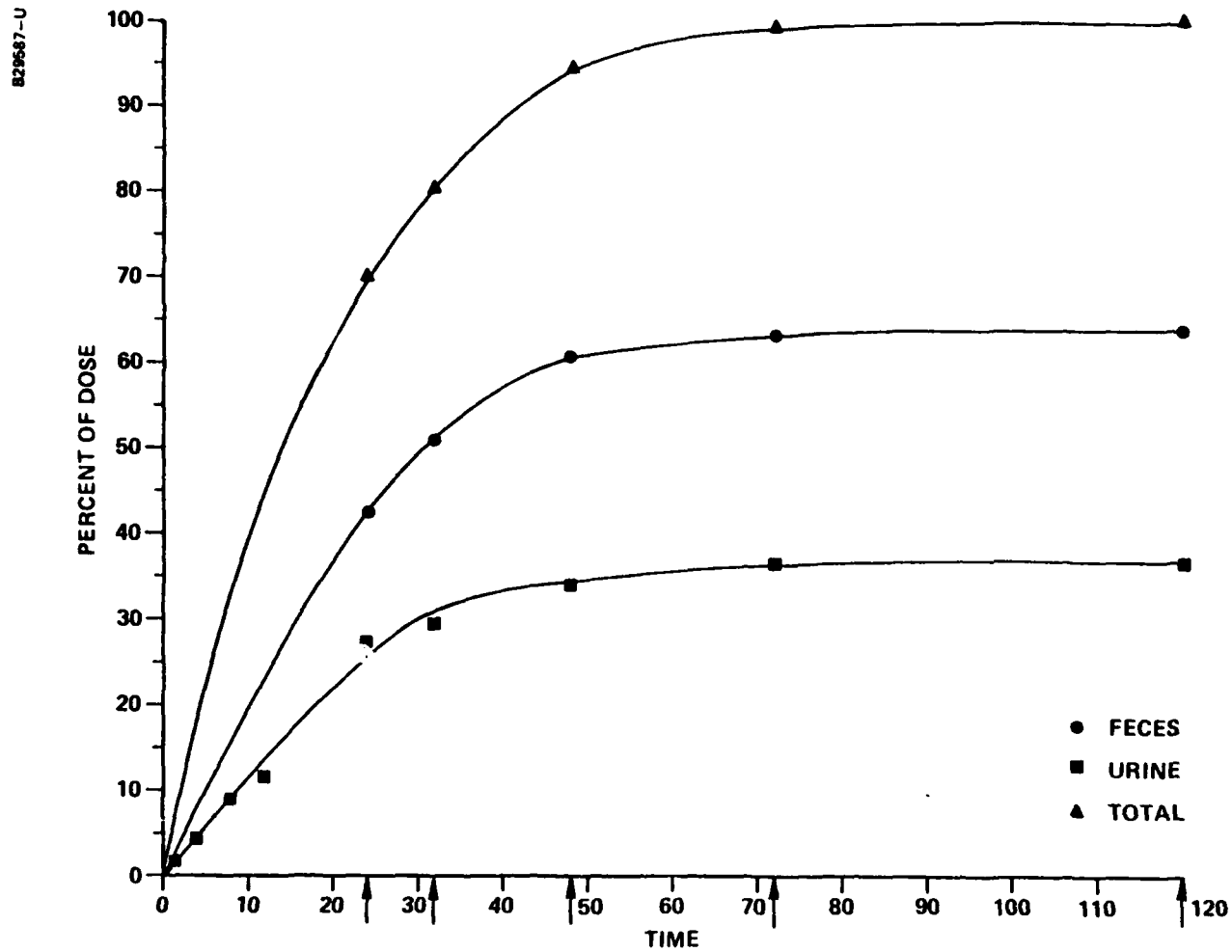

Figure 3. Cumulative excretion of radioactivity from rats administered an oral dose (160 mg/kg) of ^{14}C-PNA.

radioactivity associated with various fractions are shown in Table 2. These results show that the urine contained a high percentage of ^{14}C as materials which remained in the aqueous phase following extraction at pH 12 and 2, suggesting that PNA was extensively metabolized by the rat.

HPLC analysis of the ether extract of the urine showed at least five ^{14}C-metabolites (Figure 4). Table 3 summarizes the retention volumes and relative abundance of ^{14}C-PNA metabolites in the ether extract. No PNA could be detected in the ether extracts.

In Vitro Metabolism. In an attempt to collect sufficient amounts of PNA metabolites for characterization by spectral methods, the metabolism of PNA by the rat liver microsomes in vitro was investigated. Analysis of the ether extract of the microsomal incubation mixture by HPLC showed the presence of several metabolites. The eluant fractions corresponding to these metabolites were collected and analyzed by chemical ionization mass spectrometry (Figures 4-7). Table 4 shows the tentative structures of compounds associated with various HPLC fractions. These mixture indicate the presence of mono- and di-hydroxylated derivatives of PNA. The fractions containing both the mono and dihydroxy compounds appear to be abundantly contaminated by the corresponding quinone derivatives (P-2/e peaks in the mass spectrum), indicating further facile air oxidation of the metabolites during handling. While the position of the hydroxy group in the molecule cannot be assigned by mass spectrum alone, we infer from these results that in the mono-hydroxy derivative, the hydroxy group is in the naphthalene moiety at a position para to the amino group. In the dihydroxy derivative also, at least one hydroxy group is at the available para position in the naphthyl ring. metabolism of a structurally-related aromatic amine, diphenylamine, has been reported to give rise to 4-hydroxy- and 4,4'-dihydroxy diphenylamine (4). Based on an

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Table 2

Distribution of Radioactivity in 0-48 hr Pooled Urine Samples from Rats dosed with 160 mg/kg ^{14}C-PNA

Fraction	Percent of Total Radioactivity in the Urine
Ether extract (pH 12)	16
Ether extract (pH 2)	19
Aqueous residue (unextractable)	65

Table 3

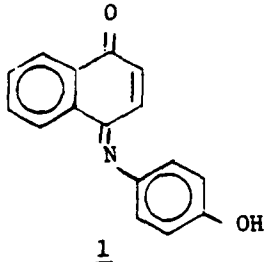
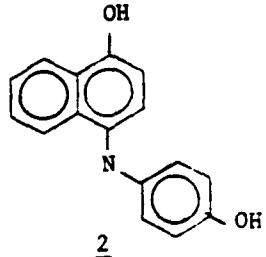
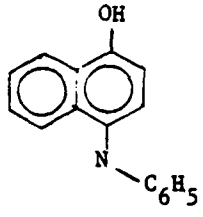
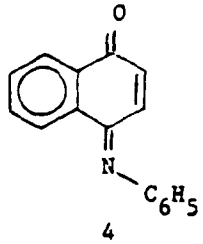
Relative Abundance of PNA Metabolies in the Urine Extracts (pH 2) from Rats Receiving 160 mg/kg ^{14}C-PNA

^{14}C-Compound		HPLC Retention Volume, ml	% of ^{14}C in the Extract
Area A	1	2.3	14
	2	3.4	20
	3	4.2	11
	4	5.8	
	5	7.3	18
	6	9.0	
Area B		12.0	23
Area C		14.5	14
PNA		28.0	ø

Figure 4. HPLC Profile of the Ether Extract of Urine from Rats Administered ^{14}C-PNA. UV Detector (---); Radioactivity Flow Detector (-).

Table 4

Mass Spectral Identification of Metabolites detected
In the Rat-Liver Microsomal System Incubated with PNA

HPLC Fraction	m/e (Chemical Ionization)	Structure
1	220, 278	A labile PNA derived product (insufficient information for structure assignment.
2	250*, 252*	 1 (Major)  2 (Minor)
3	236*, 234*	 3 (Major)  4 (Minor)
4	220	PNA

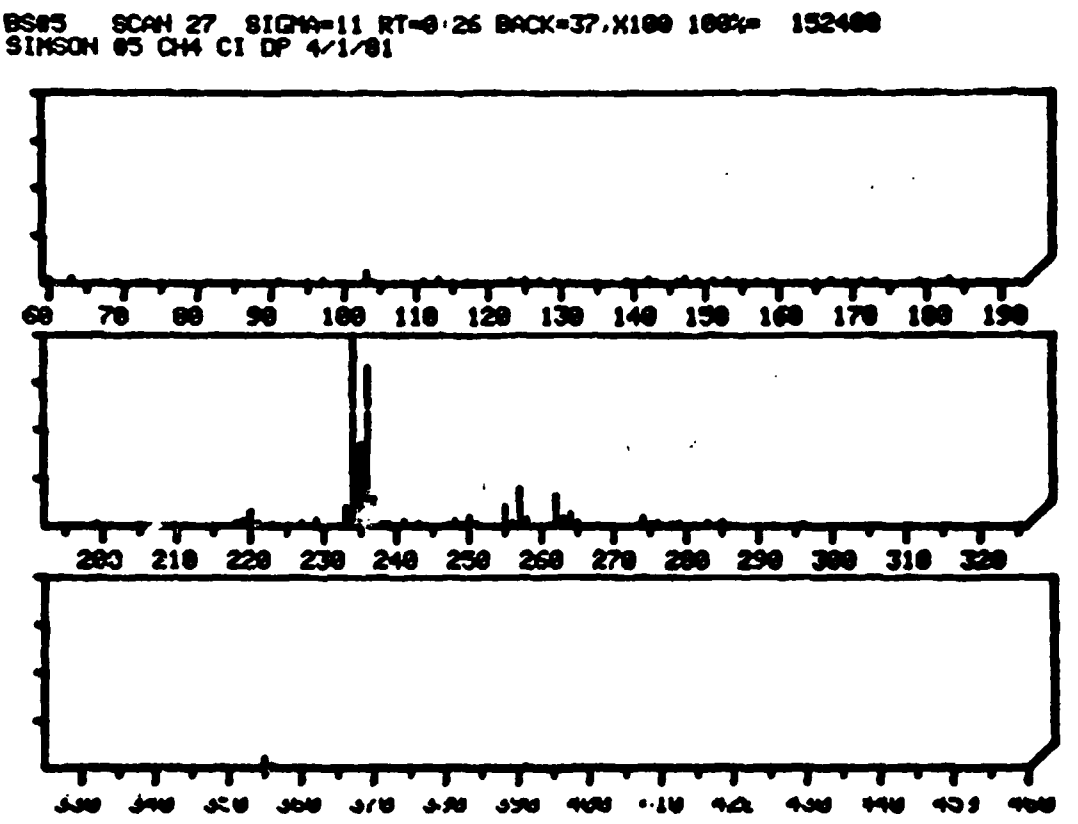


Figure 4. Mass spectrum of hydroxy PNA (3) and imino quinone (4) (mixture) isolated following incubation of PNA with rat-liver microsomes.

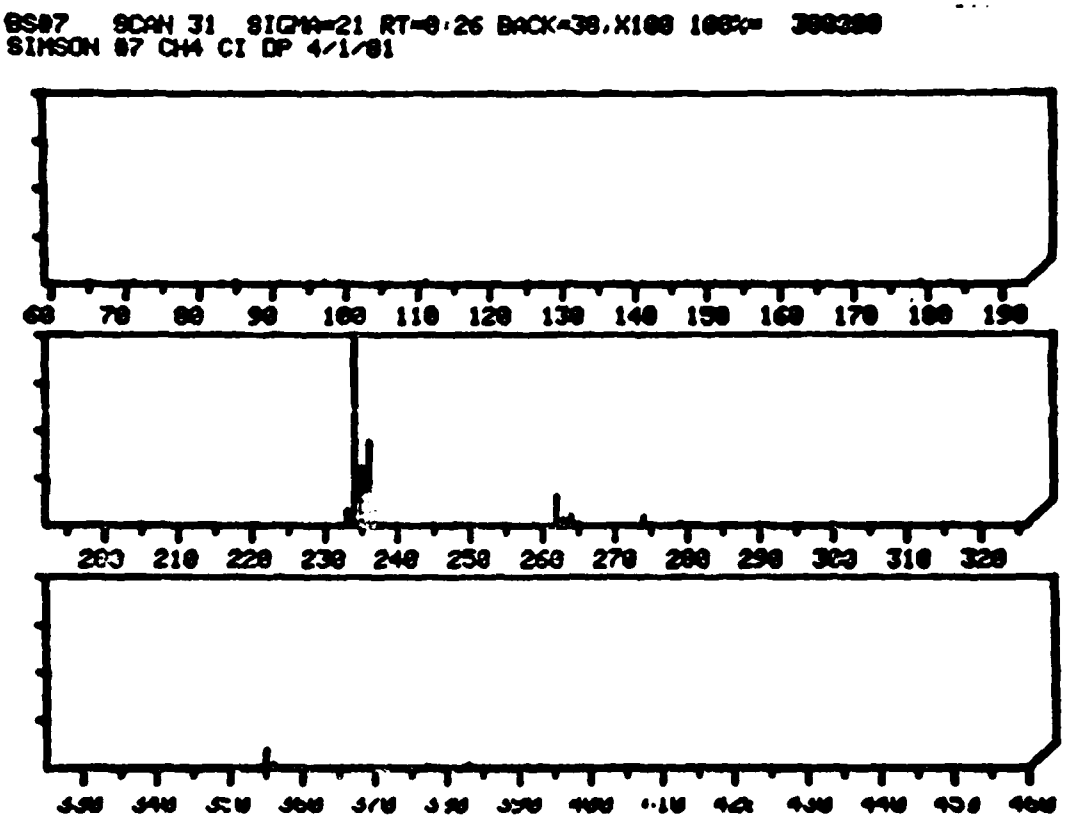


Figure 5. Mass spectrum of hydroxy PNA (3) and imino quinone (4) (mixture) isolated following incubation of PNA with rat-liver microsomes.

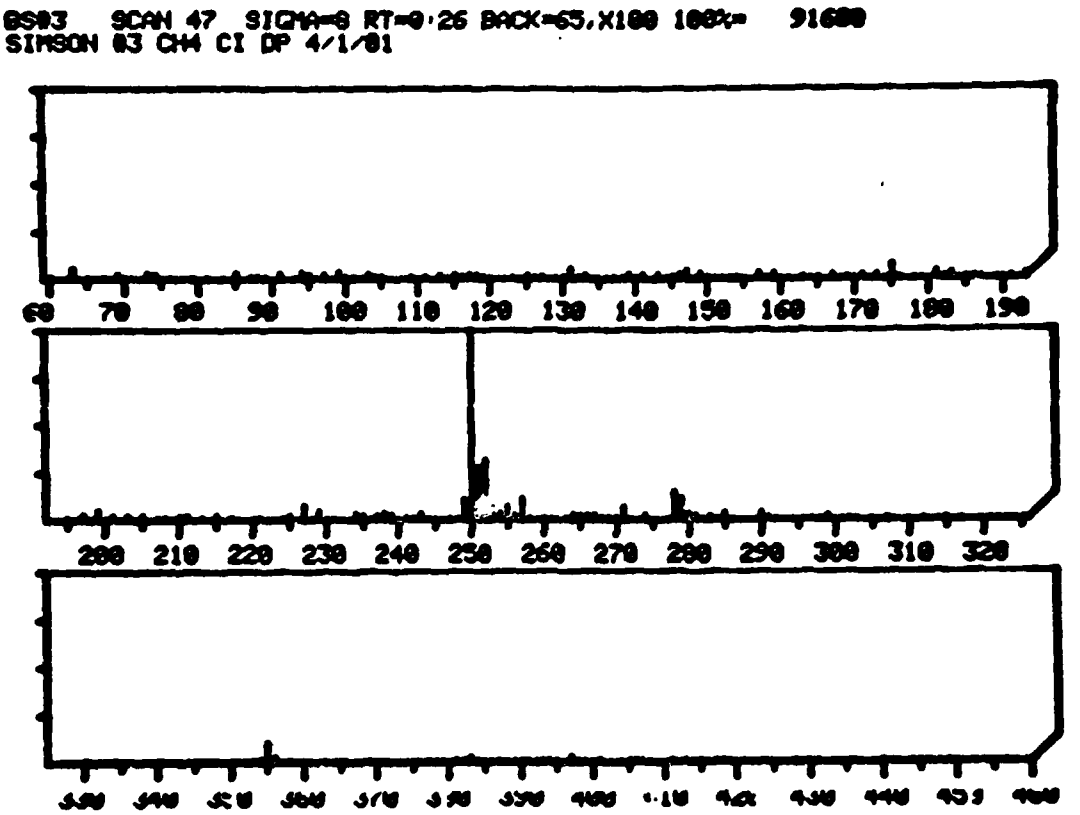


Figure 6. Mass spectrum of dihydroxy PNA (20 and hydroxy imino quinone (1) isolated following incubation of PNA with rat-liver microsomes.

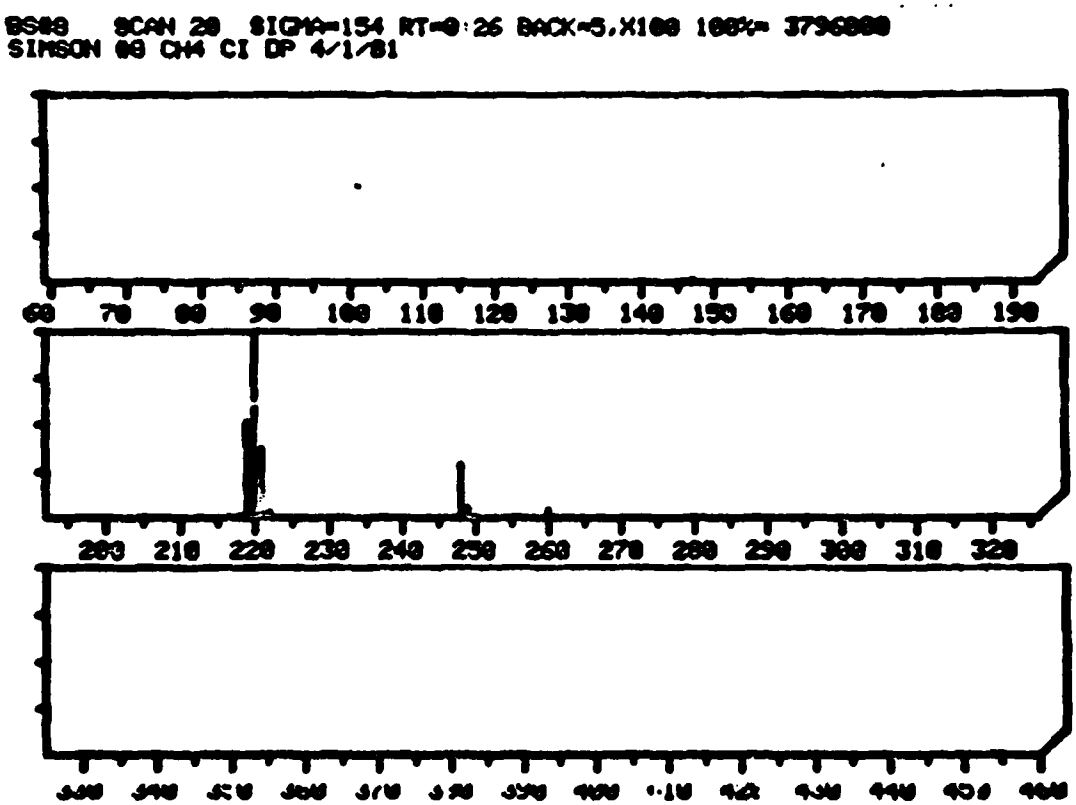


Figure 7. Mass spectrum of unreacted PNA in the rat-liver microsomal incubation mixture.

analogy to the metabolism of diphenylamine, we speculate that the dihydroxy derivatime of PNA detected in these studies is 4-hydroxyphenyl-1-(4-hydroxynaphthyl)amine. hydroxy group in each ring of the naphthalene moiety cannot be ruled out at this juncture.

DISCUSSION

The results of this investigation show that PNA is readily absorbed following oral administration as indicated by the fact that ^{14}C-PNA-derived radioactivity was detected in the blood within an hour after dosing and reached its peak in the plasma after about four hours. The level of radioactivity in the plasma followed a biphasic disappearance pattern. Such a time curve is considered to fit a two-compartment open-system model in which a central blood compartment is in reversible equilibrium with a peripheral tissue compartment (1).

PNA and/or its metabolites were excreted relatively efficiently by the rats following administration of an oral dose. During the 48 hr period following dosing, the total ^{14}C excretion amounted to about 90% of the administered dose. The ^{14}C-PNA-derived radioactivity was excreted via both the feces and urine although the primary route of excretion was via the feces, indicating biliary excretion of the chemical and/or its metabolites.

Once absorbed, PNA was rapidly distributed to most tissues of the rat. Appreciable amounts of radioactivity was detected in all the tissues examined but the principal sites of localization were the liver and fat. As with the plasma, the levels of radioactivity in these tissues declined a biphasic manner. Despite its relatively high octanol/water partition coefficient, PNA did not appear to

accumulate in the adipose tissue. This may be due to the fact that the chemical is biotransformed to metabolites with physico-chemical characteristics different from those of the parent compound.

The rat is capable of metabolizing PNA extensively. Although our data provides no information on the nature of the excretory products, the results seem to indicate that a high percentage of the PNA metabolites recovered in the urine were conjugated compounds. The results of the in vitro studies using rat-liver microsomes show that the biotransformation of PNA may involve hydroxylation in the phenyl and/or naphthyl ring. The hydroxylated materials may subsequently undergo O-glucuronidation or O-sulfation. Similar results on the metabolism of another secondary aromatic amine, diphenylamine, in the rat have been reported (4).

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